Peptidyl boronic acid inhibitors of proteasomes

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Abstract

20S proteasomes are large, multisubunit complexes that display a number of different peptidase activities. A series of di- and tripeptidyl boronic acids were shown to be tight-binding inhibitors of the liver 20S proteasome chymotrypsin-like activity. Time course experiments suggested that these peptidyl boronic acids cause inhibition of the chymotrypsin-like activity through an apparent slow-binding mechanism. Other liver 20S proteasome activites e.g. trypsin-like and PGPH were also inhibited to a lesser extent. Inhibition of 20S proteasome activities by peptidyl boronic acids was slowly reversed during dialysis. Kinetic studies showed that inhibition of the chymotrypsin-like activity only followed ideal competitive behaviour, between 0 and 2-3×Ki inhibitor concentrations. Ki values determined over these inhibitor concentrations, were between 15 and 120 nM for the most effective peptidyl boronic acids. These included peptidyl boronic acids containing Phe-boroLeu or Leu-boroLeu structures e.g. Bz-Phe-boroLeu and Cbz-Leu-boroLeu. Tri-leucine peptidyl boronic acids were shown to be the most effective inhibitors of the liver 20S proteasome trypsin-like activity. Spleen 20S proteasomes are predominantly assembled with IFN-γ-inducible catalytic subunits whereas liver 20S proteasomes are constructed with a mixture of constitutively expressed and IFN-γ-inducible catalytic subunits. Broadly similar effects were observed when the peptidyl boronic acids were tested with spleen 20S proteasomes. The most effective inhibitor of the 20S proteasome chymotrypsin-like activity, Bz-Phe-boroLeu, was also shown to potently inhibit the chymotrypsin-like activity of 26S proteasomes and 20S proteasomes activated with 0.02% SDS. Peptidyl boronic acids were found to cross the plasma membrane of cultured human or mouse cells. Because of the slow release of bound inhibitor from 20S and 26S proteasomes, it was possible to demonstrate their inhibition in immunoprecipitation experiments. 20S proteasomes, but not 26S proteasomes, remained inhibited following gel filtration of an extract prepared from cells treated with a peptidyl boronic acid. IC50 values for the chymotrypsin-like activity of 20S or 26S proteasomes immunoprecipitated from cells treated with peptidyl boronic acid, were similar to the Ki values determined for the same activity of purified 20S proteasomes.

To Bob, Joy and Neil

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AUTHOR'S DECLARATION

I declare that the work in this dissertation was carried out in accordance with the Regulations of the University of Bristol. The work is original except where indicated by special reference in the text and no part of the dissertation has been submitted for any other degree. The experiments investigating the effect of peptidyl boronic acids on the processing of the amyloid precursor protein and the activation of NF-κB, were performed by members of the neuroscience research group at SmithKline Beecham. These persons are listed as authors of the first paper in the publications arising from this work section of the thesis.

Any views expressed in the dissertation are those of the author and in no way represent those of the University of Bristol. The dissertation has not been presented to any other University for examination either in the United Kingdom or overseas.

SIGNED: R.C. Gordon DATE: 2/2/2000

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Abbreviations

Abz, 2-aminobenzoyl

AMC, 7-amino-4-methylcoumarin

β-lactone, clasto-Lactacystin β-lactone

Boc, tert-butoxycarbonyl

BrAAP, branch-chain amino acid preferring

BSA, bovine serum albumin

°C, degrees celsius

Cbz, benzyloxycarbonyl

cpm, counts per minute

DCI, 3,4-dichloroisocoumarin

DMEM, Dulbecco's modified essential medium

DMSO, dimethyl sulphoxide

EDTA, ethylenediaminetetraacetic acid

ER, endoplasmic reticulum

g, centrifugal field e.g. 1000×g

HC, MHC class I heavy chain

HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HPLC, high performance liquid chromatography

IFN, interferon

k_{obs}, observed pseudo-first-order rate constant

MHC, major histocompatibility complex

Nba, 4-nitrobenzylamide

NAP, naphthylamide

pAB, p-aminobenzoate

PBS, phosphate-buffered saline

PGPH. peptidylglutamyl-peptide hydrolase

pNA, p-nitroaniline

SBzl, thiobenzyl

SNAAP, small neutral amino acid preferring

Suc, N-succinyl

TFA, trifluoroacetic acid

Tris, tris(hydroxymethyl)aminomethane

VS, vinyl sulphone

Chapter 1 - Introduction

Introduction

1.1 - Protein degradation

The breakdown of short-lived, long-lived and abnormal/misfolded proteins occurs within all organisms. Degradation of proteins is an essential process that removes damaged and misfolded proteins. The selective degradation of proteins is also important for effective control of cellular metabolism. For example B-type cyclins bind to and modulate the activity of cyclin-dependent protein kinases. These cyclins are degraded during the metaphase-anaphase transition of the cell cycle before accumulating in the S and G_2 phases (Brandeis & Hunt, 1996). Protein degradation is thought to occur in all cellular compartments. The predominant pathway in the cytosol and nucleus is known as the ubiquitin-proteasome pathway. Ubiquitin is a 76 amino acid protein found in eukaryotic cells (Hilt & Wolf, 1996). Attachment of multiple ubiquitin chains to substrate proteins marks them for degradation by the 26S proteasome (Wilkinson, 1995; Rock et al., 1994). ATP hydrolysis is required to drive the degradation of proteins by the 26S proteasome and substrates for the pathway include the cell cycle regulators Mos, the retinoblastoma (Rb) protein and the tumor suppressor protein p53 (Ishida et al., 1993; Boyer et al., 1996; Dietrich et al., 1996). Regulators of transcription and signal tranduction are also degraded by this pathway for example, the Gα protein, protein kinase C-α, the MATα2 repressor, Gcn4, c-Jun, c-Fos and STAT1 (Hilt & Wolf, 1996; Lee et al., 1996; Tsurumi et al., 1995; Fuchs et al., 1996; Kim & Maniatis, 1996).

The substrate proteins are cut progressively, yielding 6-12 amino acid peptides. These peptides are thought to be hydrolysed to amino acids by exopeptidases (Mitch & Goldberg, 1996). 8-9 amino acid peptides displaying C-terminal hydrophobic or basic residues, are also

transported into the endoplasmic reticulum for expression on MHC class I molecules (Joyce et al., 1994; Gaczynska et al., 1993). Recent studies have suggested that the NF-κB p105 precursor protein is co-translationally processed by the 26S proteasome to give the mature p50 subunit (Lin et al., 1998b). It was not clear whether ubiquitin played any role in this reaction, that may be required for the precursor processing of other proteins.

Non-ubiquitinated proteins can also be degraded by the 26S proteasome. The best characterised example of this is the degradation of ornithine decarboxylase (ODC), a key enzyme in polyamine biosynthesis. Polyamines induce the synthesis of the protein antizyme that inhibits ODC. The association of antizyme with ornithine decarboxylase stimulates the degradation of ornithine decarboxylase by the 26S proteasome (Hayashi & Murakami, 1995; Li. et al., 1996b). The N- and C-terminal regions of antizyme were proposed to be important for interacting with 26S proteasomes and ornithine decarboxylase respectively.

1.2 - Ubiquitination of proteins

Attachment of ubiquitin molecules to substrate proteins requires the action of three types of enzyme. E1 ubiquitin-activating enzymes catalyze the formation of a thiol ester between the C-terminus of ubiquitin and a cysteine residue of the E1 enzyme. This reaction requires the expenditure of one ATP (Wilkinson, 1995). E2 ubiquitin-conjugating enzymes catalyse the transfer of ubiquitin from E1 to a cysteine residue on the E2 protein. Some larger E2 enzymes catalyse the direct transfer of ubiquitin to the substrate protein or growing ubiquitin chain. In other cases an E3 ubiquitin-ligase assists the transfer of ubiquitin from the E2 enzyme to the substrate protein (Wilkinson, 1995). The covalent linkage of ubiquitin molecules in polyubiquitin chains is usually through the C-terminal carboxyl group of one ubiquitin and the

ε-amino group of Lys-48 on the next ubiquitin. Human $E2_{EPF}$ was shown to catalyze the formation of Lys-11 linked polyubiquitin chains, in the presence or absence of an E3 ligase (Baboshina & Haas, 1996). In the absence of E3 ligase, the yeast E2 enzyme RAD6 catalysed the formation of Lys-6 linked multiubiquitin chains on the substrate protein histone H2B. In the presence of E3 ligase and using bovine serum albumin as substrate, the linkage specificity was shown to change from Lys-6 to Lys-48. Hence linkage specificity for some E2 enzymes is substrate/co-factor dependent.

Ubiquitin carboxy-terminal hydrolases (UCHs) cleave ubiquitin from e.g. peptides, lysine, glutathione or small proteins. Ubiquitin-specific processing proteases cleave ubiquitin groups from ubiquitinated proteins. Both classes of enzyme catalyze the disassembly of polyubiquitin chains (Wilkinson, 1995; Wilkinson, 1997). The UCH enzymes cleave the covalent bond between the undegraded peptide and the C-terminus (proximal end) of the polyubiquitin chain (Hochstrasser, 1995; Wilkinson, 1995). Ubiquitin-specific processing proteases e.g. Ubp14p in yeast or isopeptidase T in mammalian cells, then release single ubiquitin molecules from the proximal end of the ubiquitin chain (Wilkinson, 1997).

1.3 - The 20S proteasome - the catalytic core of the 26S proteasome

The 26S proteasome is a large multisubunit complex which is constructed from the 20S proteasome and two 19S regulatory particles that fit onto the ends of the 20S proteasome (Demartino et al., 1996). The 20S proteasome is a 700 kDa complex, composed of four seven-membered subunit rings. These rings are stacked together to form a barrel-shaped structure approximately 11 nm in diameter and 15 nm in length (Hegerl et al., 1991; Wenzel & Baumeister, 1995; Kopp et al., 1995). The Thermoplasma acidophilum 20S proteasome is

composed of two subunits. The molecular masses of the α and β subunits are 26 and 23 kDa respectively. Immuno-electron microscopy in conjunction with subunit-specific antibodies, have been used to show that the outer protein rings of the proteasome are composed of α-subunits. The two juxtaposed inner rings are composed of β-subunits (Grziwa et al., 1991). The central channel of the 20S proteasome has three large cavities. Two of the cavities are located at the interface between the α and β rings, and the third is between the β rings (Löwe et al., 1995). The minimum diameter of the cavity is 13Å which means that only unfolded polypeptides can pass through the channel.

Thermoplasma α-subunits expressed in the absence of β subunits, spontaneously form into heptameric rings that did not possess peptidase activity (Zwickl et al., 1994). Loose aggregates of mature β subunits, expressed in the absence of α subunits, were shown to exhibit moderate peptidase activity compared to 20S proteasomes. β precursor proteins expressed in the absence of α-subunits exhibited virtually no proteolytic activity. Hence it was proposed that autocatalytic processing of the β-subunits occured during 20S proteasome assembly. This would ensure that the catalytic activity of the β-subunits was not activated, until the proteasome was assembled (Zwickl et al, 1994).

1.3.1 - The structure of eukaryotic 20S proteasomes closely resembles prokaryotic 20S proteasomes

Yeast 20S proteasomes are composed of 14 different subunits that have molecular weights of 20-34 kDa (Heinemeyer et al., 1994). These subunits are encoded by genes of a single gene family. The subunits can be divided into two groups, based on their similarity to the

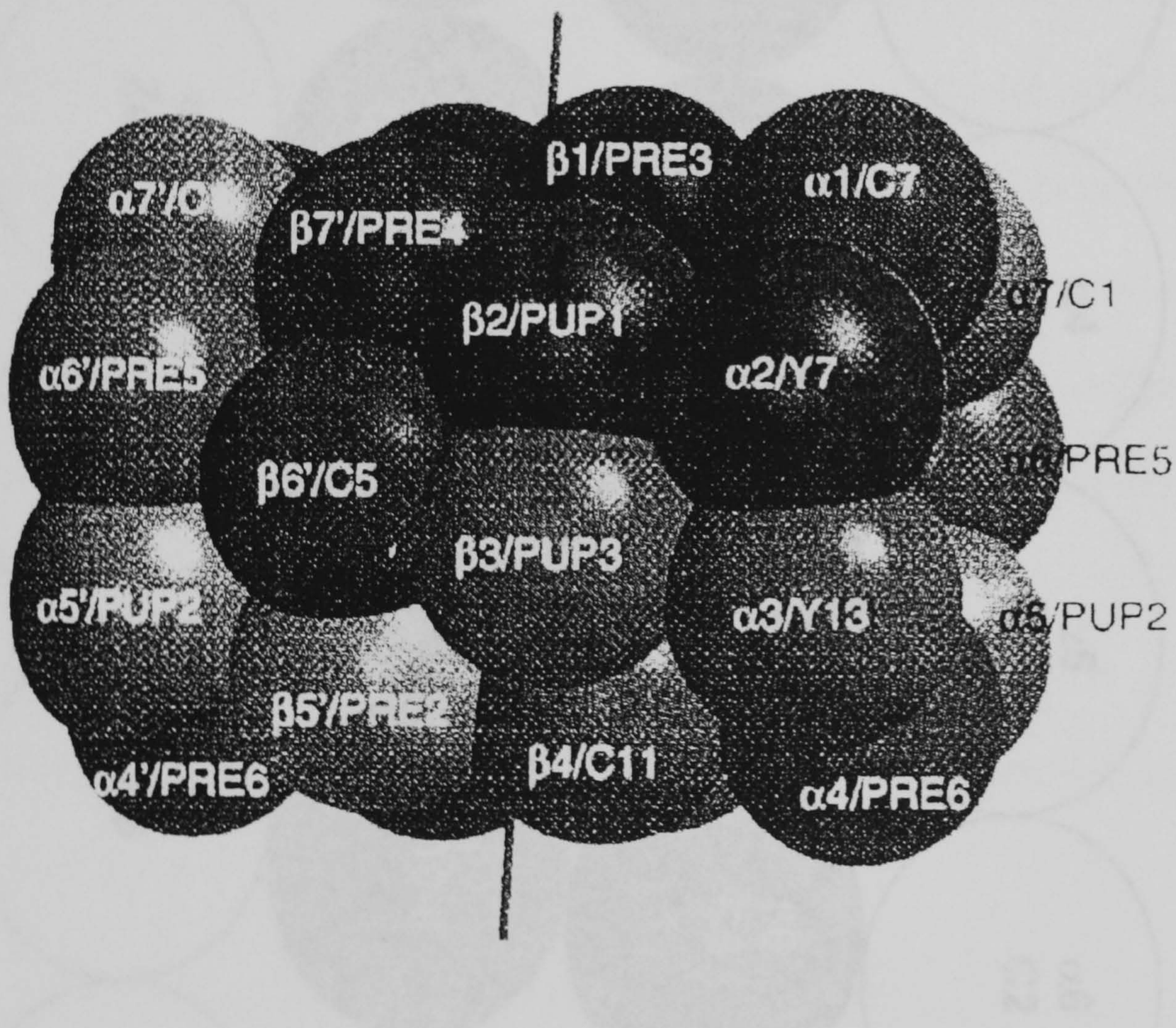
archaebacterial α or β subunits. The two outer rings of the complex are composed of α-type subunits. The two juxtaposed inner rings of the complex are composed of β-type subunits (Groll et al., 1997). Figure 1.1A shows the arrangement of subunits in the yeast 20S proteasome. Higher eukaryotic organisms express 7 α-type and 10 β-type subunits with molecular weights of 20-34 kDa (Hilt & Wolf, 1996; Hisamatsu et al., 1996). Figure 1.1B shows the arrangement of subunits in the human 20S proteasome. The expression of the mammalian β subunits LMP2, LMP7 and MECL1 is upregulated by the cytokine IFN-γ (Hisamatsu et al., 1996). Several studies have shown that increased expression of IFN-γ inducible subunits, causes a greater proportion of these subunits to be incorporated into new 20S proteasomes (Hisamatsu et al., 1996; Früh et al., 1994; Akiyama et al., 1994; Belich et al., 1994). This occurs at the expense of three constitutively expressed β-subunits Y/δ, X/MB1 and Z, that exhibit a high degree of identity to the IFN-γ inducible subunits. Experimental data did not suggest that IFN-γ directly suppressed the expression of the constitutive proteins. Instead it was proposed that the IFN-γ inducible subunits were preferentially incorporated into newly synthesized 20S proteasomes (Früh et al., 1994; Belich et al., 1994).

The crystal structure of the yeast 20S proteasome shows the entrance to the inner chambers, being blocked by the N-terminal regions of five α-type subunits α1/C7, α2/Y7, α3/Y13, α6/PRE5 and α7/C1. Substantial movements of these subunits were proposed to be required for entry of unfolded polypeptides (Groll et al., 1997). These movements may occur during the ATP-dependent attachment of regulatory particles to the ends of the 20S proteasome. Narrow (~10Å diameter) openings in the side of the complex, particularly near the interface between the α- and β-rings were observed. Small peptide substrates and longer unfolded polypeptides may gain access to the catalytic sites through these entrances (Groll et al, 1997).

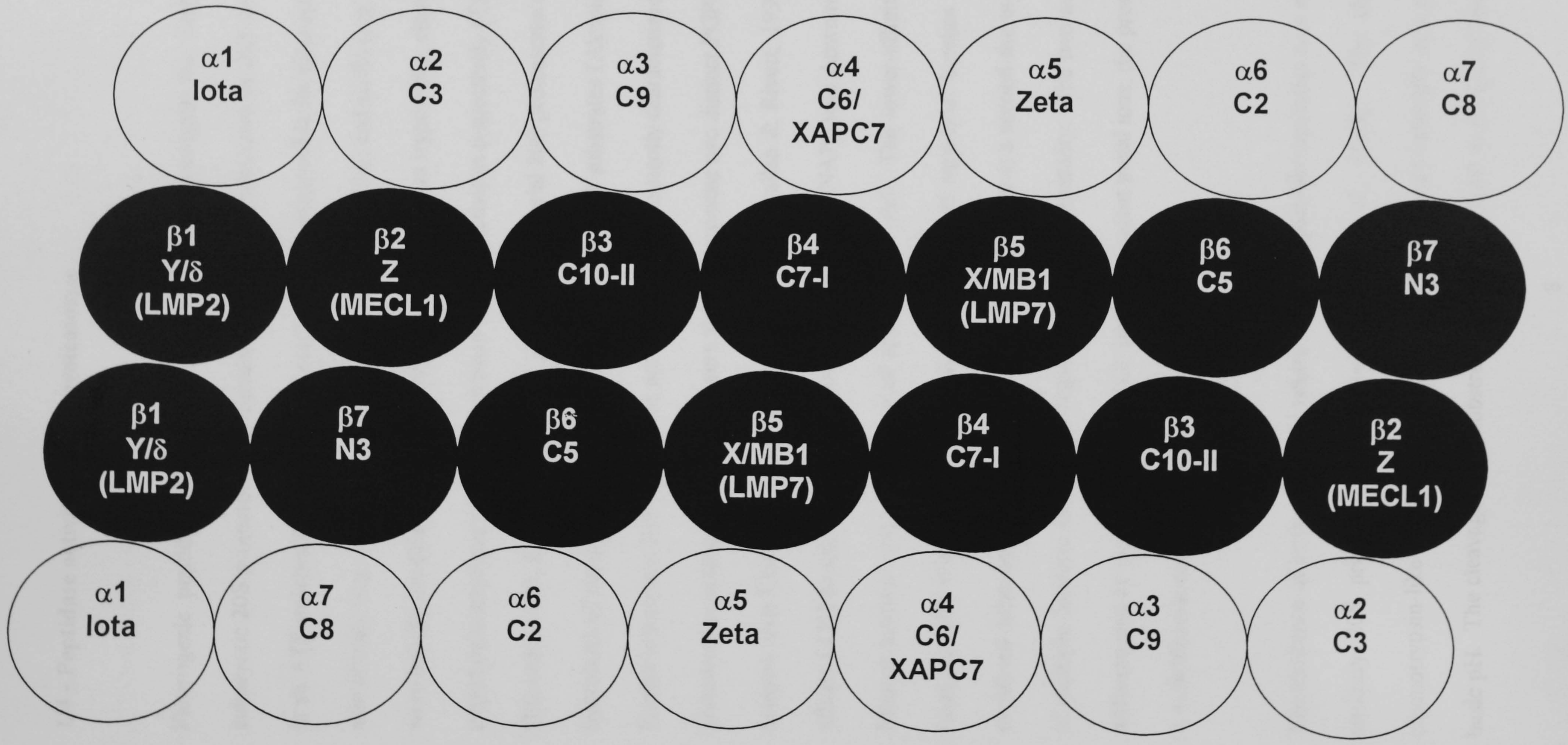
Figure 1.1 - Arrangement of subunits in yeast and human 20S proteasomes

Panel A illustrates the arrangement of subunits in yeast 20S proteasomes determined through the crystallisation of the complex. The figure was reproduced from the report of Groll et al. (1997). Panel B illustrates the arrangement of subunits in human 20S proteasomes as determined by Kopp et al. (1997) and Dahlmann et al. (1999). The IFN-γ inducible subunits LMP7, LMP2 and MECL1 are incorporated into the complex in place of X/MB1, Y/δ and Z respectively.

Panel A



Panel B


α1
Iota
α2
C3
α3
C9
α4
C6/
XAPC7
α5
Zeta
α6
C2
α7
C8
β1
Y/δ
(LMP2)
β2
Z
(MECL1)
β3
C10-II
β4
C7-I
β5
X/MB1
(LMP7)
β6
C5
β7
N3
β1
Y/δ
(LMP2)
β7
N3
β6
C5
β5
X/MB1
(LMP7)
β4
C7-I
β3
C10-II
β2
Z
(MECL1)
α1
Iota
α7
C8
α6
C2
α5
Zeta
α4
C6/
XAPC7
α3
C9
α2
C3

1.4 - Peptidase activities of 20S proteasomes

Fluorogenic peptide substrates have been used to investigate the catalytic activites of eukaryotic 20S proteasomes. Degradation of peptide substrates by 20S proteasomes occurs in an ATP-independent manner. Distinct activities responsible for cleavage on the carboxyl side of basic (e.g. Arg), hydrophobic (e.g. Leu, Tyr and Phe) and acidic (e.g. Glu) amino acids were initially identified. These activities were referred to as trypsin-like, chymotrypsin-like and peptidylglutamyl-peptide bond hydrolase (PGPH) activities respectively (Table 1.1) (Wilk & Orlowski, 1980; Rivett et al., 1994). The archaebacterial 20S proteasome also catalyses the hydrolysis of chymotrypsin-like, trypsin-like and PGPH substrates (Akopian et al., 1997). Further eukaryotic 20S proteasome activities were subsequently characterised (Table 1.1). 20S proteasomes isolated from rat liver were shown to possess two distinct PGPH activities when assayed with Cbz-LLE-β-naphthylamide (NAP) (Djaballah & Rivett, 1992). The higher-affinity LLE1 activity (assayed with 0-0.1 mM Cbz-LLE-NAP), was shown to obey Michaelis-Menten kinetics with a K_m value of approx. 100 μM. The lower-affinity LLE2 activity (assayed with 0.4-0.6 mM Cbz-LLE-NAP) exhibited sigmoidal kinetics. This could be explained either by allosteric activation of the proteinase at a second site or the involvement of multiple catalytic sites which display positive cooperativity. The two activities could be differentiated by their response to KCl, selected divalent metal ions, the proteasome inhibitor 3,4-dichloroisocoumarin (DCI) and heat treatment.

The existence of both acidic and neutral chymotrypsin-like activities was shown for bovine pituitary 20S proteasomes (Figueiredo-Pereira et al., 1995). The cleavage of most chymotrypsin-like substrates e.g. Suc-LLVY-AMC occurs optimally at a neutral or slightly basic pH. The cleavage of the substrate Boc-VEAL- NAP at the L-NA bond was shown to

occur optimally at pH 5.5 and 45°C. The two chymotrypsin-like activities could be differentiated by their responses to sodium and calcium ions and selected proteasome inhibitors e.g. Cbz-GGL-al.

Two other chymotrypsin-like activities have been described for bovine pituitary 20S proteasomes (Orlowski et al., 1993). The first of these activities was named 'branch-chain amino acid preferring' (BrAAP) that preferentially cleaves on the carboxyl side of branched chain amino acids e.g. leucine. The BrAAP activity was stimulated by DCI that inhibits the chymotrypsin-like activity. The second activity, named 'small neutral amino acid preferring' (SNAAP) preferentially cleaves on the carboxyl side of small neutral amino acids e.g. glycine and alanine. This activity was not inhibited by the aldehyde inhibitors chymostatin and Cbz-LLF-al but was inhibited by DCI and N-ethylmaleimide. Further work showed that the identity of the residues preceding the cleavage site e.g. third (P3) residue from the cleavage site, regulates the passage of a substrate to a particular chymotrypsin-like active site (Cardozo et al., 1994). Cardozo and coworkers (1996) showed that the BrAAP activity caused hydrolysis of substrates on the carboxyl side of Pro and Glu residues. The cleavages were assigned to the BrAAP activity on the basis of inhibition by the BrAAP inhibitor Cbz-GPFL-al, competition by a BrAAP substrate and activation of both cleavages by DCI. These results show that the BrAAP activity exhibits a broad P1 specificity.

Table 1.1 - Peptidase activities of the 20S proteasome

Peptidase activity	Position of cleavage in Typical substrates
Chymotrypsin-like	↓ Suc-LLVY-AMC ↓ AAF-AMC ↓ Cbz-GGL-AMC ↓ Cbz-GGL-pNA
Acidic chymotrypsin-like	↓ Boc-VEAL-NAP
Branch-chain amino acid preferring (BrAAP)	↓ Cbz-GPAL-G-pAB ↓ Cbz-GPAL-A-pAB ↓ Abz-GPAL-A-Nba
Small neutral amino acid preferring (SNAAP)	↓ Cbz-GPAG-G-pAB ↓ Cbz-GPAA-G-pAB
Trypsin-like	↓ Boc-LSTR-AMC ↓ Cbz-D(A)LR-NAP ↓ Cbz-GGR-AMC ↓ Cbz-ARR-AMC
Peptidylglutamyl peptide hydrolase (PGPH) LLE1 activity	↓ Cbz-LLE-NAP ↓ Cbz-LLE-pNA
LLE2 activity	↓ Cbz-LLE-NAP ↓ Boc-AAD-SBzl

Abbreviations: Suc, N-succinyl; AMC, 7-amino-4-methylcoumarin; Cbz, benzyloxycarbonyl; pNA, p-nitroaniline; Boc, tert-butoxycarbonyl; NAP, naphthylamide; pAB, p-aminobenzoate; Abz, 2-aminobenzoyl; Nba, 4-nitrobenzylamide; SBzl, thiobenzyl.

The hydrolysis of the leaving group in these substrates is generally monitored by measuring the fluorescence of the solution at defined excitation and emission wavelengths or through a diazotization procedure (Rivett et al., 1994; Orlowski et al., 1993). Cleavage of the pAB group in the BrAAP and SNAAP substrates is usually very slow unless an aminopeptidase e.g. aminopeptidase N is present in the assay solution.

1.5 - Protease inhibitors have been used to gain a greater understanding of the 20S proteasome activities

A variety of protease inhibitors and effectors have been employed to study 20S proteasome activities. In one of the first studies on 20S proteasomes a number of compounds e.g. p-chloromercuribenzoate, N-ethylmaleimide, iodoacetamide and inorganic salts such as $ZnCl_2$ and NaCl caused inhibition of peptidase activities (Wilk & Orlowski, 1980). Most of these compounds caused inhibition of the chymotrypsin-like activity with some inhibiting the trypsin-like and PGPH activities. Similar results were obtained in other studies using e.g. palmitic acid but these inhibitors were used at millimolar concentrations and gave no clear indication of the number or type of catalytic sites being affected (Orlowski & Michaud, 1989; Dahlmann et al., 1985). A number of serine protease inhibitors were tested against mammalian 20S proteasome activities. One of these was 3,4-dichloroisocoumarin (DCI) that causes irreversible inhibition of serine proteases e.g. human leukocyte elastase, cathepsin G and human factor D (Harper et al., 1985). Several studies showed that DCI was most effective at inhibiting the chymotrypsin-like activity measured with the substrates Cbz-GGL-pNA, Cbz-GGF-pNA, AAF-AMC or Suc-LLVY-AMC (Table 1.2; pages 16 and 17) (Orlowski & Michaud, 1989; Cardozo et al., 1992; Orlowski et al., 1993; Orlowski et al., 1997; Djaballah et al., 1992). The SNAAP, trypsin-like and PGPH activities of 20S proteasomes were inhibited using suitable concentrations of DCI (Orlowski et al., 1993; Orlowski et al., 1997). Low concentrations of DCI sometimes caused stimulation of the trypsin-like and PGPH activities (Djaballah et al., 1992; Orlowski et al., 1993). For example 20 μM DCI stimulated the hydrolysis of 0.1 mM Cbz-LLE-NAP (Djaballah & Rivett, 1992). Stimulation of these activities could result from inhibition of one type of catalytic site, causing conformational changes that stimulate the activity at other sites. DCI was also shown to stimulate the BrAAP activity of bovine pituitary

proteasomes (Orlowski et al., 1993).

Relatively high (5 mM) concentrations of the serine protease inhibitor diisopropyl fluorophosphate (DFP), inhibited the chymotrypsin-like activity of 20S proteasomes measured with the Cbz-GGL-pNA and Suc-LLVY-AMC substrates (Orlowski & Michaud, 1989; Djaballah et al., 1992). This irreversible inhibitor was also proposed to inhibit the trypsin-like and PGPH activities in one study (Djaballah et al., 1992). These results are in contrast to Mason (1990) who proposed that 25 mM DFP did not inhibit the Cbz-GGR-AMC activity of liver 20S proteasomes.

Peptidyl chloromethanes and peptidyl diazomethanes irreversibly inhibit serine or cysteine proteases by modifying active site residues (Table 1.2) (Mason, 1990). YGR-CH_2Cl was found to be an effective inhibitor of the liver 20S proteasome chymotrypsin-like and trypsin-like activities (Reidlinger et al., 1997). AAF-CH_2Cl and Cbz-FGY-CHN_2 were shown to inhibit the 20S proteasome chymotrypsin-like activity. Other amino acid combinations gave rise to less effective inhibitors (Savory et al., 1993; Mason, 1990). It was observed that either YGR-CH_2Cl or AAF-CH_2Cl inhibited the hydrolysis of the chymotrypsin-like substrate Suc-LLVY-AMC, to a much greater extent than AAF-AMC using either liver 20S or 26S proteasomes (Reidlinger et al., 1997). This effect was also observed in another report investigating the antitumor drug Aclacinomycin A (Figueiredo-Pereira et al., 1996). Aclacinomycin A (100 μM) inhibited the 20S proteasome chymotrypsin-like activity measured with Suc LLVY-AMC or Cbz-IEAL-pNA by 36% and 45% respectively. The hydrolysis of another chymotrypsin-like substrate Cbz-GGL-pNA was inhibited by 26% using the same concentration of Aclacinomycin A (Figueiredo-Pereira et al., 1996). These differences in inhibition may indictate that each chymotrypsin-like substrate is hydrolysed to some extent by

other catalytic sites. The nature of the P2, P3 and P4 residues as well as P1 will affect which catalytic site(s) predominantly hydrolyse the substrate (Stein et al., 1996).

1.5.1 - Peptidyl aldehydes are effective inhibitors of 20S proteasome activities

Peptidyl aldehydes have proved to be one of the most effective classes of 20S proteasome inhibitor. In one study Cbz-PGAL-al was shown to be the most effective inhibitor of the chymotrypsin-like activity measured with the Cbz-GGL-pNA substrate (Vinitsky et al., 1994). The Ki value for inhibition of this activity was 16.4 μM with corresponding Ki values of 36.7 μM for the SNAAP activity (cleavage of Cbz-GPAGG-pAB at the G-G bond) and 104 μM for the BrAAP activity (cleavage of Cbz-GPALG-pAB at the L-G bond). Another inhibitor Cbz-GPFL-al was most effective against the BrAAP and SNAAP activities with Ki values of 1.5 and 2.3 μM respectively. Ac-LLnL-al or Cbz-LLnV-al effectively inhibited the rabbit 20S proteasome chymotrypsin-like and PGPH activities (Rock et al., 1994). The Ki values for inhibition of Suc-LLVY-AMC hydrolysis were 0.14 and 0.021 μM for Ac-LLnL-al and Cbz-LLnV-al respectively. The corresponding Ki values for inhibition of Cbz-LLE-NAP hydrolysis were 1.2 and 0.66 μM. These Ki values were obtained using an assay buffer containing 0.035% SDS. SDS (0.01-0.02%) is known to activate the chymotrypsin-like and PGPH activities of 20S proteasomes (Rivett, 1989; Arribas & Castaño, 1990). The trypsin-like activity was either inhibited or stimulated depending on the substrate used and the precise assay conditions. SDS was proposed to stimulate the peptidase activities by inducing conformational changes and greater conformational flexibility in the complex. Higher concentrations of SDS e.g. 0.1% inhibited the peptidase activities of 20S proteasomes (Arribas & Castaño, 1990). Rock and coworkers (1994) determined similar Ki values for the effect of

Ac-LLnL-al and Cbz-LLnV-al on the rabbit 26S proteasome chymotrypsin-like and PGPH activities. This confirmed that the catalytic activities of both 20S and 26S proteasomes were effectively inhibited by the peptidyl aldehydes.

Peptidyl aldehydes have played an important role in defining and specifically inhibiting the activities of mammalian 20S proteasomes. The hydrolysis of Boc-VEAL-NAP at the L-NAP bond was found to occur optimally at pH 5.5 (Figueiredo-Pereira et al., 1995). This is in contrast to other chymotrypsin-like substrates e.g. Cbz-GGL-NAP that are optimally hydrolysed in neutral or slightly basic buffers. Cbz-GGL-al (286 μM) was shown to inhibit the hydrolysis of Cbz-GGL-NAP by 84%. The same concentration of inhibitor caused only 14% inhibition of the acidic chymotrypsin-like activity measured with Boc-VEAL-NAP. Cbz-LLE-al and Cbz-GPFL-al were used in one report to show that the 20S proteasome BrAAP activity can cleave after Pro and Glu residues (Cardozo et al., 1996). This study increased the known substrate specificity of the BrAAP activity from the previously defined post-Leu cleavage. Leupeptin (Acetyl-LLR-al) has been shown to specifically inhibit the trypsin-like activity of mammalian 20S proteasomes (Wilk & Orlowski, 1980; Savory & Rivett, 1993). Hydrolysis of the chymotrypsin-like substrates Cbz-GGL-pNA, Cbz-GGYA-pNA, Cbz-GGYS-NAP, AAF-AMC or the PGPH substrate Cbz-LLE-NAP was not significantly inhibited, using leupeptin concentrations that caused substantial inhibition of the trypsin-like activity measured with Cbz-D(A)LR-NAP, Bz-FVR-pNA or Boc-LSTR-AMC.

Table 1.2 - Categories of proteasome inhibitor

Type of compound	General comments	References
Isocoumarin derivatives e.g. 3,4-dichloroisocoumarin (DCI)	Selective irreversible inhibitor of serine proteases; micromolar e.g. 50 μM DCI causes irreversible inhibition of the chymotrypsin-like and SNAAP activities and stimulation of the BrAAP activity; inhibits or stimulates the trypsin-like and PGPH activities depending on concentration used and assay conditions.	Orlowski & Michaud, 1989; Djaballah et al., 1992; Orlowski et al., 1997.
Peptidyl diazomethanes e.g. Cbz-FGY-CHN_2	Irreversible inhibitors of serine and cysteine proteases; compounds are irreversible inhibitors of the chymotrypsin-like activity; other activities are less effectively inhibited; effects depend on the amino acids in the compound; need to be used at e.g. 100 μM concentrations.	Savory et al., 1993; Reidlinger et al., 1997.
Peptidyl chloromethanes e.g. YGR-CH_2Cl, AAF-CH_2Cl	Irreversible inhibitors of serine and cysteine proteases; compounds irreversibly inhibit the chymotrypsin-like, trypsin-like and PGPH activities; effects depend on the amino acids in the compound; generally used at 10-100 μM concentrations.	Savory et al., 1993; Reidlinger et al., 1997.

Peptidyl aldehydes e.g. Ac-LLnL-al, Cbz-LLnV-al (MG115), Cbz-LLL-al (MG132)	Compounds cause reversible inhibition of chymotrypsin-like, acidic chymotrypsin-like, trypsin-like, PGPH, BrAAP and SNAAP activities; some compounds are potent inhibitors of the chymotrypsin-like activity with e.g. 0.02 μM Ki values; compounds inhibit calpains and cathepsin B.	Rock et al.,1994 Vinitsky et al., 1994.
Lactacystin and clasto-lactacystin β-lactone	Lactacystin inhibits the chymotrypsin-like, trypsin-like, PGPH and BrAAP activities; chymotrypsin-like activity is most effectively inhibited; the inhibition is slowly reversed; clasto-lactacystin β-lactone is readily formed from lactacystin at pH 8 and is the sole species that inhibits proteasomes; lactacystin inhibits a platelet cathepsin A-like activity; generally used at 10-100 μM concentration	Fenteany et al., 1995; Dick et al., 1996.
Peptidyl vinyl sulphones e.g. Cbz-LLL-$(CH)_2SO_2Me$ (Z-LLL-VS)	Irreversible inhibitors of chymotrypsin-like, trypsin-like and PGPH activities; effects depend on the amino acids in the compound; one compound NIP-LLL-VS is known to inhibit cathepsin S; generally used at 0.1-100 μM concentration	Bogyo et al., 1997; Bogyo et al., 1998.

1.5.2 - Lactacystin - a particularly specific proteasome inhibitor

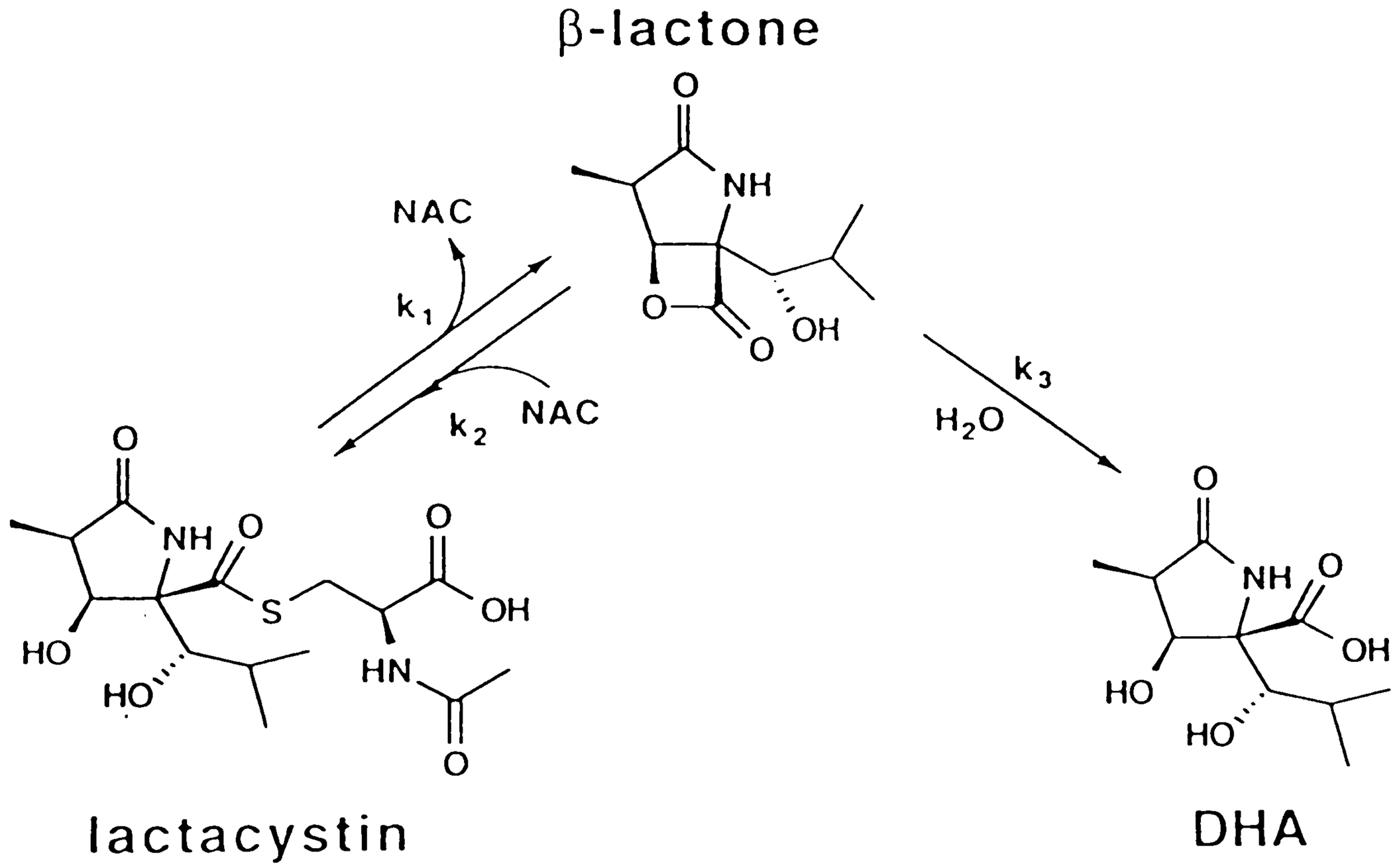
Peptidyl aldehydes have been used in many in vivo studies, to imply a role of proteasome complexes in the degradation of proteins. Cbz-LLL-al (MG-132) in particular has been used to imply a role of 26S proteasomes in degrading the cystic fibrosis transmembrane conductance regulator (CFTR) protein and the retinoblastoma (Rb) protein. Inhibition of proteasome complexes by Cbz-LLL-al was also proposed to lead to the the induction of heat shock proteins (Jensen et al., 1995; Boyer et al., 1996; Lee & Goldberg, 1998). However, peptidyl aldehydes are known to inhibit other proteases e.g. calpains and cathepsins (Rock et al., 1994). Investigators have attempted to overcome this problem by using less effective peptidyl aldehydes and other protease inhibitors to show that effective inhibition of proteasome complexes correlates with effective inhibition of protein degradation (Palombella et al., 1994).

Lactacystin is a streptomyces metabolite that inhibits the cell cycle and induces neurite outgrown in neuroblastoma cells (Fenteany et al., 1995). Figure 1.2 shows the structure of lactacystin and the mechanism by which it decays to clasto-lactacystin β-lactone (β-lactone) and N-acetyl-L-cysteine. The β-lactone is then slowly hydrolysed to a dihydroxy acid (DHA). Experiments in which [^{3}H] lactacystin or [^{3}H] β-lactone were incubated with crude extracts of Neuro-2a cells or bovine brain, showed that 20S proteasomes were the only radiolabelled cellular target (Fenteany et al., 1995). The chymotrypsin-like activity of purified 20S proteasomes was effectively inhibited by 10 μM lactacystin or 1 μM β-lactone. The k_{obs}/I values measured with the Suc-LLVY-AMC substrate were 194 and 3059 $M^{-1}s^{-1}$ respectively (Fenteany et al., 1995). Lactacystin exhibited k_{obs}/I values of 10 and 4 $M^{-1}s^{-1}$ for the trypsin-like and PGPH activities. Lactacystin was initially thought to irreversibly inhibit the catalytic

activities of 20S proteasomes. However, later studies showed that the inhibition was slowly reversed, at a rate that was dependent on the source of 20S proteasomes (McCormack et al., 1997). Results from another report showed that β-lactone was the sole species that inhibited proteasome activities (Dick et al., 1996). Conditions that stabilised lactacystin and inhibited the production of β-lactone, greatly reduced the effectiveness of lactacystin as a proteasome inhibitor. Although lactacystin is a remarkably specific inhibitor of proteasomes, a recent study showed that 1-5 μM lactacystin could inhibit a human platelet cathepsin A-like activity (Ostrowska et al., 1997). This inhibition was dependent on incubation of lactacystin at pH 8.0 for 10 minutes to allow the production of the β-lactone species, before conducting the assays at pH 5.5.

Figure 1.2 - Mechanism of aqueous hydrolysis of lactacystin

The streptomyces metabolite lactacystin breaks down to clasto-lactacystin β-lactone (β-lactone) and N-acetyl-L-cysteine (NAC) (Dick et al., 1996). The reaction occurs rapidly at pH 8.0 but reducing the pH to 6.3 caused lactacystin to become stable. The β-lactone is the sole species that inhibits the 20S proteasome. The β-lactone is slowly hydrolysed to the inactive dihydroxy acid (DHA). The figure is reproduced from the report of Dick et al. (1996).



Recent studies have developed a new substrate 2-aminobenzyl-(Abz)-GPALA-4-nitrobenzylamide-(Nba) for assaying the BrAAP activity of mammalian 20S proteasomes (McCormack et al., 1998). Cleavage of this substrate occurs exclusively at the L-A bond to yield Abz-GPAL and A-Nba products. The fluorescence of the Abz group in the first product can be used to follow the reaction, without needing to use an aminopeptidase to release the pAB group of other BrAAP substrates (Table 1.1) (Orlowski et al., 1993). Inhibition of the 20S proteasome BrAAP activity by compounds with the general structure Cbz-LL-Xaa-al, followed Michaelis kinetics when Xaa was Leu or norVal. Biphasic kinetic curves were observed if Xaa was Phe, Trp or (p-Cl)Phe (McCormack et al., 1998). The peptidyl aldehydes that displayed simple kinetics were relatively potent inhibitors of the 20S proteasome chymotrypsin-like and PGPH activities. In contrast the peptidyl aldehydes that exhibited biphasic kinetics were effective inhibitors of the chymotrypsin-like activity and poor inhibitors of the PGPH activity. The effect of β-lactone on the BrAAP activity was assessed in an assay using 20S proteasomes and saturating amounts of the PA28 activator. This hexameric complex binds to the 20S proteasome and activates the chymotryptic, tryptic and PGPH activities (Kuehn & Dahlmann, 1996). The results suggested that 2.5 mol β-lactone/mol 20S proteasome inhibited 90% of the chymotrypsin-like activity and also inhibited 60% of the BrAAP activity. In further experiments the chymotrypsin-like activity of 20S proteasomes was selectively inhibited using a suitable concentration of β-lactone. The Ki values for inhibition of the BrAAP or PGPH activites by e.g. Cbz-LLL-al or Cbz-LLE-al, were under these circumstances identical. It was therefore proposed that the BrAAP activity was catalysed by the active sites responsible for the chymotrypsin-like and PGPH activities (McCormack et al., 1998). This conclusion was not supported by Orlowski et al. (1993) who suggested that DCI caused inhibition of the chymotrypsin-like and PGPH activities whilst stimulating the BrAAP activity.

1.6 - The active site nucleophile of 20S proteasomes is a threonine residue

Early inhibitor studies from several groups suggested that the active site nucleophiles of 20S proteasomes were either serine or cysteine residues (Rivett, 1989, Mason, 1990). Alignment of Thermoplasma and human β-subunit sequences suggested that a conserved Ser-129 or one of two conserved threonine residues Thr-1 and Thr-2 could be the active site nucleophile (Seemüller et al., 1995). The Ser-129 residue could be mutated without impairing the catalytic activity of the resulting 20S proteasomes. In contrast deletion of Thr-1 and Thr-2 or their mutation to Ala, yielded 20S proteasomes that were correctly folded and assembled but entirely inactive (Seemuller et al.,1995). Further experiments showed that deletion of Thr-1 was sufficient to eliminate catalytic activity. In addition the mutation of Thr-1 to Ser yielded fully active 20S proteasomes that were 15 times more sensitive to DCI. A conserved Lys-33 residue was proposed to play the proton acceptor/donor role in substrate hydrolysis (Seemuller et al.,1995).

Analysis of Thermoplasma 20S proteasome crystals produced in the presence of Ac-LLnL-al identified the amino-terminal threonine as the catalytic nucleophile (Löwe et al., 1995). Ac-LLnL-al was bound to all 14 β-catalytic subunits with the aldehyde functional group located at the amino-terminal threonine. Lys-33 and Glu-17 were also located close to Thr-1 suggesting that Glu-17 may assist the proton acceptor/donor role of Lys-33. The α and β subunits were shown to fold in a very similar manner, with the notable exception of the extra N-terminal HO helix of α-subunits. This helix is important in the assembly of 7-membered α-subunit rings and may play a role in interacting with regulatory complexes of the 20S proteasome (Löwe et al., 1995).

Analysis of yeast 20S proteasome crystals showed that Lys-33 and Asp-17 were close to Thr-1 of the catalytic β-subunits β1/PRE3, β2/PUP1 and β5/PRE2 (Groll et al., 1997; Dick et al., 1998). Yeast 20S proteasome crystals were subsequently prepared in the presence of Ac-LLnL-al or lactacystin. The Ac-LLnL-al aldehyde group was covalently bound to the hydroxyl group of Thr-1 for all three catalytic β-subunits (Groll et al., 1997). Lactacystin was shown to covalently bind to the β5/PRE2 Thr-1 residue. The mammalian homologue of β5/PRE2 is X/MB1 that was shown to be labelled by 10 μM [^{3}H] lactacystin (Fenteany et al., 1995). In subsequent experiments 100 μM [^{3}H] lactacystin was shown to label all six mammalian catalytic β-subunits X/MB1, Y/δ, Z, LMP7, LMP2 and MECL1 of 20S and 26S proteasomes (Craiu et al., 1997). The arrangement of residues in the β5/PRE2 catalytic site, suggested that chymotrypsin-like and trypsin-like substrates were likely to be hydrolysed by this site (Groll et al., 1997). The β1/PRE3 site was proposed to catalyse the PGPH activity. The β2/PUP1 site was predicted to exhibit a broad substrate specificity.

In subsequent work, two separate groups have mutated yeast catalytic threonine residues to alanine to determine which peptidase activities are catalysed at the different sites (Dick et al., 1998; Chen & Hochstrasser, 1996; Arendt & Hochstrasser, 1997). Mutation of the β5/PRE2 Thr-1 to Ala or Lys-33 to Ala completely inhibited the chymotrypsin-like activity of purified 20S proteasomes, measured with Cbz-GGL-AMC or Cbz-GGL-pNA. The trypsin-like activity measured with e.g. Cbz-ARR-AMC or Cbz-ARR-4-methoxy-NAP was the same as wild-type 20S proteasomes. The PGPH activity measured with Cbz-LLE-NAP was either the same or greater than wild-type 20S proteasomes. Similar experiments linked mutation of the β2/PUP1 Thr-1 to Ala with complete loss of the trypsin-like activity. The near complete loss of PGPH activity was observed with 20S proteasomes containing the β1/PRE3 Thr-1 to Ala mutant

subunit (Dick et al., 1998; Arendt & Hochstrasser, 1997). The mutations in either β2/PUP1 or β1/PRE3 caused partial inhibition or stimulation of other proteasome activities. This suggested that mutation of one catalytic site causes conformational changes that effect substrate hydrolysis at other catalytic sites. It was also proposed that stimulation of activity was due to overexpression of 20S proteasomes in mutant cells compared to wild-type cells (Arendt & Hochstrasser, 1997). The BrAAP activity of wild-type and mutant 20S proteasomes was investigated using the Cbz-GPALA-pAB substrate (Dick et al., 1998). These experiments suggested that the BrAAP activity was catalysed by the β1/PRE3 catalytic site that also catalyses the PGPH activity. β1/PRE3 Thr-1 to Ala mutant 20S proteasomes were almost completely devoid of BrAAP activity measured with the chosen substrate.

1.6.1 - Proteasome complexes are members of the N-terminal nucleophilic hydrolase family of enzymes

Comparison of the subunit secondary structure of 20S proteasomes, the cysteine protease glutamine PRPP amidotransferase and the serine protease penicillin acylase suggest that these enzymes belong to a new family named N-terminal nucleophilic hydrolases or Ntn hydrolases (Groll et al., 1997; Duggleby et al., 1995; Brannigan et al., 1995). The catalytic mechanism of penicillin acylase is proposed to involve a water molecule, that is hydrogen bonded to the α-amino group of the catalytic Ser-1 residue. The water molecule in conjunction with the α-amino group are thought to perform the proton acceptor/donor role in the catalytic mechanism. The arrangement of a water molecule hydrogen bonded to the α-amino group of Thr-1, has been suggested as a proton acceptor/donor in 20S proteasome catalytic sites (Groll et al., 1997; Brannigan et al., 1995). However, yeast 20S proteasomes exhibiting the β5/PRE2 Lys-

33 to Ala mutation were devoid of chymotrypsin-like activity (Dick et al., 1998). This result clearly indicated that the potential proton acceptor/donor residue Lys-33 is required for substrate hydrolysis.

1.6.2 - Use of peptidyl vinyl sulphones to link mammalian 20S proteasome activities to particular catalytic sites

Peptidyl vinyl sulphones have recently been employed to irreversibly inhibit the peptidase activities of recombinant Rhodococcus and mammalian 20S proteasomes (Bogyo et al., 1997; McCormack et al., 1997). Different combinations of amino acids give rise to compounds that preferentially inhibited one or more of the proteasome activities. For example 4-hydroxy-3-iodo-2-nitrophenyl acetyl-LLL-$(CH)_2SO_2Me$ (NLVS) inhibited the 20S proteasome chymotrypsin-like, trypsin-like and PGPH activities with k_{obs}/I values of 13500, 10 and 24 $M^{-1}s^{-1}$ respectively (Bogyo et al., 1998). LLLL-vinyl sulphone (VS) was a relatively specific inhibitor of the trypsin-like activity. A k_{obs}/I value of 1,500 $M^{-1}s^{-1}$ was substantially larger than values of 240 for the chymotrypsin-like activity and 29 for the PGPH activity. Peptidyl vinyl sulphones are reported to be remarkably specific for proteasome complexes. [^{125}I] NLVS was able to enter three different human cells and label either proteasome catalytic subunits or a protease that was identified as cathepsin S (Bogyo et al., 1997). The major labelled proteasome β-subunits were LMP2, LMP7 and X/MB1. Proteasomes in yeast or Thermoplasma acidophilum cell extract could also be labelled. [^{125}I] NLVS labelled mutant Thermoplasma 20S proteasomes composed of Thr-1 to Ser β-subunits. Other mutant Thermoplasma 20S proteasomes composed of Thr-1 to Ala β subunits were not labelled.

In one study the k_{obs}/I values for inhibition of the 20S proteasome chymotrypsin-like, trypsin-

like and PGPH activities were determined for 11 peptidyl vinyl sulphones (Bogyo et al., 1998). Radiolabelled forms of some of the peptidyl vinyl sulphones were synthesized and used to label proteasomes in total cell lysates. The proteasome complexes were immunoprecipitated and then separated by SDS-PAGE, 2-D SDS-PAGE or non-equilibrium pH gradient electrophoresis followed by SDS-PAGE in the second dimension (Table 1.3). [^{125}I] NLVS was shown to modify the LMP7 and X/MB1 subunits to a much greater extent than [^{125}I] YLLL-VS. NLVS displayed a 9 times higher k_{obs}/I value for inhibition of the chymotrypsin-like activity measured with Suc-LLVY-AMC, compared to YLLL-VS. The relatively weak chymotrypsin-like inhibitor LLLL-VS, competed poorly against [^{125}I] YLLL-VS for binding to the LMP7 and X/MB1 subunits when compared to the more effective chymotrypsin-like inhibitors NLVS and Bz-FLLL-VS. Hence LMP7 and X/MB1 were proposed to predominantly catalyse the chymotrypsin-like activity. This assignment is in agreement with Reidlinger and coworkers (1997) who showed that [^{3}H] acetyl-AAF-CH_2Cl primarily inhibited the chymotrypsin-like activity of liver 20S and 26S proteasomes. This inhibitor was shown to predominantly label subunits LMP7 and X/MB1 of 20S proteasomes. Also lactacystin preferentially inhibits the chymotrypsin-like activity of 20S proteasomes. [^{3}H] lactacystin (10 μM) was shown to label subunit X/MB1 of 20S proteasomes (Fenteany et al., 1995). The six mammalian catalytic subunits were labelled by 100 μM [^{3}H] lactacystin in a separate study (Craiu et al., 1997). Studies using [^{14}C] DCI and a number of peptidyl aldehydes also concluded that inhibition of the pituitary 20S proteasome chymotrypsin-like activity was linked to labelling of subunit X/MB1 (Orlowski et al., 1997). However, this group suggested that inhibition of the spleen 20S proteasome chymotrypsin-like activity was associated with modification of the LMP2 subunit. The LMP7 subunit was tentatively assigned to the BrAAP activity although other studies have proposed that this activity is not catalysed by a separate site (McCormack et al., 1998; Dick et al., 1998).

Table 1.3 - Assignment of peptidase activities to mammalian catalytic β-subunits

[a] - Results from Bogyo et al. (1998)

[b] - Results from Reidlinger et al. (1997)

[c] - Results from Fenteany et al., (1995)

[d] - Results from Orlowski et al., (1997)

[e] - Results from McCormack et al., (1998)

Peptidase activity	Assigned β-subunits	Basis for assignment
Chymotrypsin-like	LMP7, X/MB1 (LMP2)	a) [^{125}I] NLVS modified LMP7 and X/MB1 subunits to a much greater extent than [^{125}I]-YLLL-VS. NLVS displayed a 9 times higher k_{obs}/I value for the chymotrypsin-like activity compared to YLLL-VS [a]. b) The relatively weak chymotrypsin-like inhibitor LLLL-VS competed poorly against [^{125}I]-YLLL-VS for binding to LMP7 and X/MB1. The more effective chymotrypsin-like inhibitor NLVS was a better competitor for LMP7 and X/MB1 binding in similar experiments [a]. c) [^{3}H] acetyl-AAF-CH_2Cl primarily inhibits the chymotrypsin-like activity of 20S and 26S proteasomes. This inhibitor predominantly labelled subunits LMP7 and X/MB1.[b] c) Lactacystin is much more effective at inhibiting the chymotrypsin-like activity than the trypsin-like or PGPH activities. [^{3}H] Lactacystin preferentially modifies the X/MB1 subunit [c]. d) Studies using [^{14}C] DCI and peptidyl aldehydes suggested that the chymotrypsin-like activity was catalysed by X/MB1 and LMP2 subunits [d].

Trypsin-like	MECL1, Z	a) [^{125}I] Y-LLL-VS modified MECL1 and Z subunits to a much greater extent than [^{125}I] NLVS. Y-LLL-VS displayed a 56 times higher k_{obs}/I value for the trypsin-like activity compared to NLVS [a]. b) The more effective trypsin-like inhibitors LLLL-VS and Bz-FLLL-VS, competed with [^{125}I] Y-LLL-VS for binding to MECL1 and Z subunits, to a greater extent than YLLL-VS or NLVS [a].
PGPH (LLE1)	LMP2, Y/δ	a) [^{125}I] GLLL-VS-PhOH exclusively labelled the LMP2 and Y/δ subunits. This inhibitor displayed relatively low k_{obs}/I values for the chymotrypsin-like and trypsin-like activities [a]. b) NLVS displayed a similar k_{obs}/I value to GLLL-VS-PhOH for the PGPH activity. [^{125}I] NLVS also labelled the LMP2 subunit to a similar level as [^{125}I] GLLL-VS-PhOH [a].
PGPH (LLE2)	Y/δ	a) Studies using [^{14}C] DCI and peptidyl aldehydes suggested that the PGPH activity measured with 0.64 mM Cbz-LLE-NAP was catalysed by Y/δ [d].
BrAAP	a) LMP7 b) LMP7, X/MB1, LMP2, Y/δ	a) Studies using [^{14}C] DCI and peptidyl aldehydes lead to the tentative suggestion that the BrAAP activity was catalysed by the LMP7 subunit [d]. b) Studies using lactacystin and peptidyl aldehydes suggested that the BrAAP activity was catalysed by the active sites responsible for the chymotrypsin-like and PGPH activities [e].
SNAAP	-	
Acidic chymotrypsin-like	-	

[^{125}I]-YLLL-VS was shown to modify the MECL1 and Z subunits to a much greater extent than [^{125}I] NLVS. YLLL-VS also displayed a 56 times higher k_{obs}/I value for inhibition of the trypsin-like activity measured with Boc-LRR-AMC, compared to NLVS. The most effective trypsin-like inhibitors were LLLL-VS and Bz-FLLL-VS with k_{obs}/I values of 1,500 and 2,370 $M^{-1}s^{-1}$ compared to 560 $M^{-1}s^{-1}$ for YLLL-VS. LLLL-VS and Bz-FLLL-VS were able to compete more efficiently against [^{125}I]-YLLL-VS for labelling of the MECL1 and Z subunits, when compared to YLLL-VS and NLVS. Hence MECL1 and Z were proposed to predominantly catalyse the trypsin-like activity.

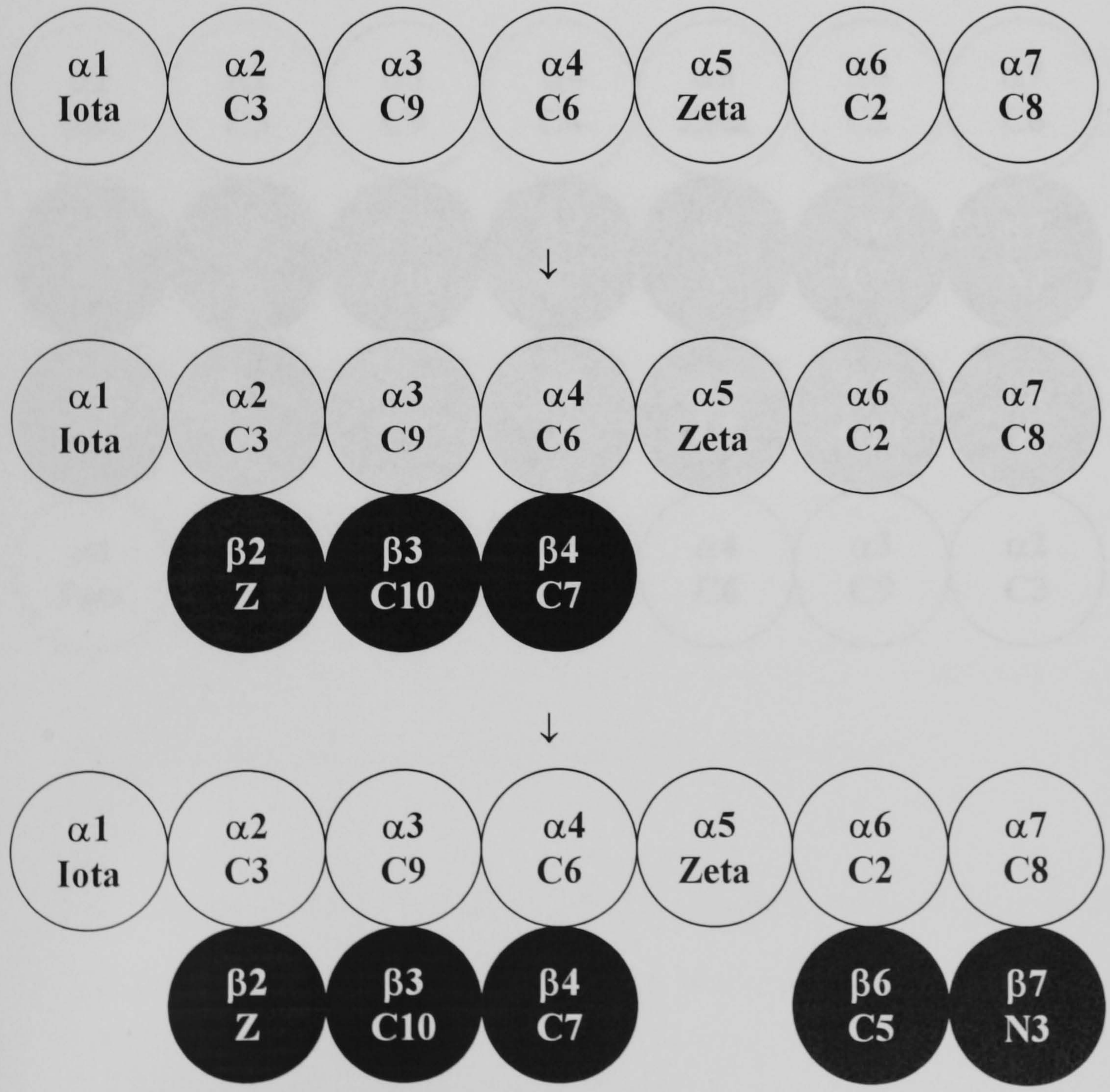
[^{125}I]-GLLL-VS-PhOH was shown to exclusively label the LMP2 and Y/δ subunits. The unlabeled form of this inhibitor displayed a relatively low k_{obs}/I value for the chymotrypsin-like and trypsin-like activities. In contrast the k_{obs}/I value for the PGPH activity was comparable to several other vinyl sulphones. NLVS displayed a similar k_{obs}/I value to GLLL-VS-PhOH for the PGPH activity. In addition [^{125}I] NLVS labelled the LMP2 subunit to a similar extent as [^{125}I] GLLL-VS-PhOH. Therefore LMP2 and Y/δ were proposed to predominantly catalyse the PGPH activity. Orlowski and coworkers (1997) also linked inhibition of the PGPH activity with modification of the Y/δ subunit. This group measured the PGPH activity of 20S proteasomes using 0.64 mM Cbz-LLE-NAP. Hence the Y/δ subunit may be responsible for the LLE2 activity defined by (Djaballah & Rivett, 1992). This lower affinity activity, distinct from the higher affinity LLE1 activity, was proposed to be responsible for the cleavage of the Cbz-LLE-NAP substrate at high e.g. 0.64 mM substrate concentrations.

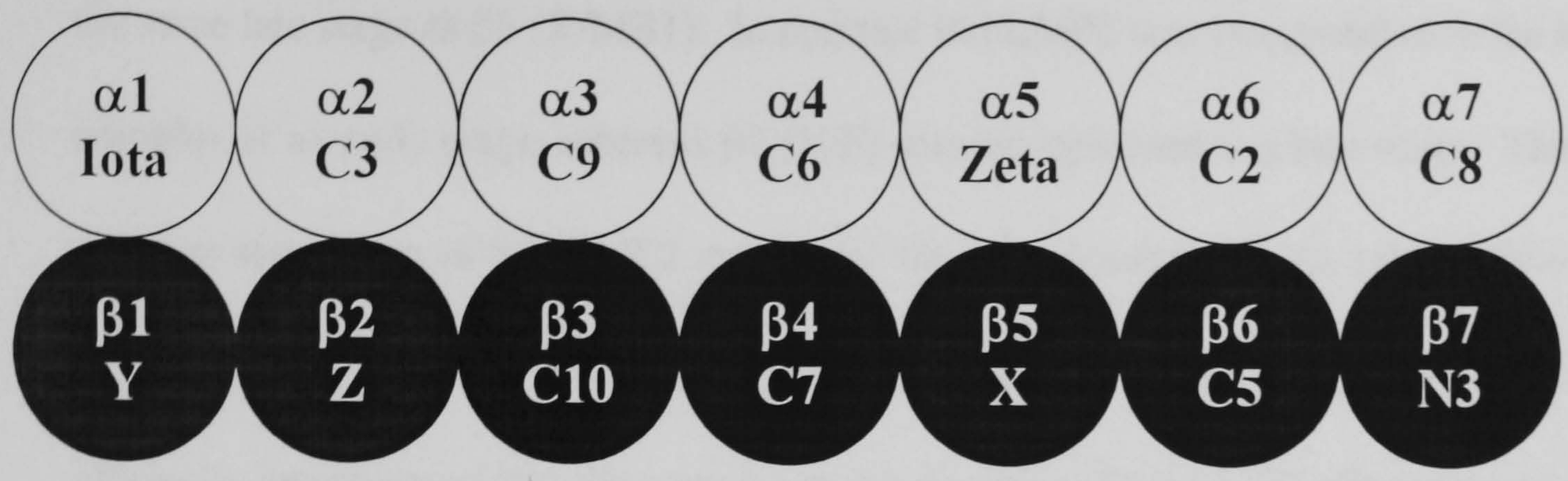
1.7 - Assembly of eukaryotic 20S proteasomes

Archaebacterial 20S proteasome α-subunits spontaneously assemble into 7-membered rings that associate with β precursor proteins in the first stage of assembly (Zwickl et al., 1994). Eukaryotic α-type subunits are proposed to associate together to form heptameric rings, containing all seven α-type subunits in a specified arrangement (Figures 1.1A and 1.1B) (Groll et al., 1997; Schmidt & Kloetzel, 1997). The β-subunits β4/C7-I and β3/C10-II along with the β2/Z proprotein are thought to associate with the α-subunit rings to form the 13S intermediate complex (Frentzel et al., 1994; Nandi et al., 1997). α-subunits are synthesized as mature subunits. The β4/C7-I and β3/C10-II subunits are not synthesized with a prosequence and do not have an active site N-terminal threonine. The prosequence of β2/Z is slowly autoprocessed by the catalytic Thr-1 residue (Schmidt & Kloetzel, 1997) (Figure 1.3). The β6/C5 and β7/N3 proproteins, neither of which contain an active site, are then incorporated with the active subunits β5 (X/MB1) and β1 (Y/δ) being added last to yield the 16S intermediate complex (Schmidt & Kloetzel, 1997). Two of the 16S 'half proteasome' complexes are suggested to associate together to form a complex dimer, where final processing of the β-propeptides occurs (Schmidtke et al., 1997; Kopp et al., 1993). Processing of β6/C5, β7/N3, β5 (X/MB1) and β1 (Y/δ) was proposed to occur via a trans-autocatalytic reaction, in which active subunits cleave the propeptides of other active and inactive (β6/C5 and β7/N3) subunits (Thomson & Rivett, 1996; Schmidt & Kloetzel, 1997). Molecular chaperones e.g. hsc73, Ump1p are present in the intermediate complexes and are proposed to assist the autocatalytic processing of the large (up to 80 residues) propeptides (Schmidtke et al., 1997; Ramos et al., 1998). These chaperone molecules are not incorporated into the final 20S proteasome structure.

Figure 1.3 - Assembly of eukaryotic 20S proteasome complexes

In the first stage the α-subunits associate together in a specified arrangement to form seven membered rings (See Figure 1.1A and 1.1B). The β4/C7-I and β3/C10-II subunits that are not synthesized with a prosequence and do not have a N-terminal threonine are then incorporated together with the prosequence of β2/Z. This prosequence is thought to be slowly autoprocessed by the catalytic Thr-1 residue (Schmidt & Kloetzel, 1997). The β6/C5 and β7/N3 proproteins, neither of which contain a catalytic threonine residue, are then incorporated into the 13S complex with the active subunit proproteins β5 (X/MB1) and β1 (Y/δ) being added last to yield the 16S intermediate complex (Schmidt & Kloetzel, 1997). Two of the 16S 'half proteasome' complexes are suggested to associate together to form a complex dimer, where final processing of the β-propeptides occurs (Schmidtke et al., 1997; Kopp et al., 1993).




α1 Iota
α2 C3
α3 C9
α4 C6
α5 Zeta
α6 C2
α7 C8
β1 Y
β2 Z
β3 C10
β4 C7
β5 X
β6 C5
β7 N3
2×
α1 Iota
α2 C3
α3 C9
α4 C6
α5 Zeta
α6 C2
α7 C8
β1 Y
β2 Z
β3 C10
β4 C7
β5 X
β6 C5
β7 N3
β1 Y
β7 N3
β6 C5
β5 X
β4 C7
β3 C10
β2 Z
α1 Iota
α7 C8
α6 C2
α5 Zeta
α4 C6
α3 C9
α2 C3

The inducible subunit β2i/MECL1 was reported to be incorporated into the assembling complex at the same early stage as β2/Z (Nandi et al., 1997). β5i/LMP7 was incorporated at the same late stage as β5 (X/MB1). In contrast β1i/LMP2 was incorporated in the assembling complex at an early stage, whereas β1 (Y/δ) was incorporated at a late stage. Therefore the early incorporation of β1i/LMP2 may assist the incorporation of the other IFN-γ inducible subunits. This proposal was supported by the fact that β5i/LMP7 and β2i/MECL1 are only efficiently incorporated into the complex in the presence of β1i/LMP2 (Groettrup et al., 1996a; Kuckelkorn et al., 1995).

1.7.1 - Assembly of eukaryotic 26S proteasomes

The 26S proteasome is formed by the association of two 700 kDa regulatory particles with the ends of the 20S proteasome. These complexes, also known as the 19S cap complex or PA700 complex are composed of 17-18 different subunits (Walz et al., 1998; Glickman et al., 1998). Six of these subunits are ATPases of the AAA (ATPases associated with various cellular activities) ATPase family. Studies in which His_6-tagged versions of the ATPase subunits were expressed in yeast cells, suggested that each regulatory particle contains all six ATPases (Glickman et al., 1998). A model in which the ATPase subunits associate together in a six-membered heteromeric ring has been proposed (Walz et al., 1998). This ATPase ring may associate with the α-subunit ring and play an important role in regulating the passage of unfolded polypeptides, to the catalytic sites of the 20S proteasome.

One of the human non-ATPase subunits, S5a, binds specifically to the polyubiquitin chains of ubiquitinated lysozyme. S5a was also shown to bind to polyubiquitin chains containing four

or more ubiquitin molecules (Deveraux et al., 1994). S5a and the yeast homologue SUN1/MCB1 are proposed to exist free in the cytosol, presumably binding to ubiquitinated proteins and directing them to 26S proteasomes (Tanaka & Chiba, 1998). Disruption of the SUN1/MCB1 gene did not cause lethality in yeast which suggested that other polyubiquitin receptor (pUB-R) proteins may be expressed. Recent studies had identified at least five different human pUB-R proteins that are generated from a single gene by alternative splicing (Tanaka & Chiba, 1998). The non-ATPase subunit Pad1p has been suggested to catalyse the isopeptidase activity associated with the 26S proteasome (Lam et al., 1997). Both Pad1p and the yeast homolog Rpn11 are thought to exhibit isopeptidase activity, because they display sequences e.g. close to a possible cysteine nucleophile, that are conserved in deubiquitinating enzymes (Glickman et al., 1998; Wilkinson et al., 1997).

The association of regulatory particles with 20S proteasomes occurs through an ATP dependent mechanism and causes activition of 20S proteasome activities (Chu-Ping et al., 1994). Three-dimensional electron microscopy studies have suggested that the linkage between the regulatory particles and the 20S proteasome is flexible, allowing a limited degree of movement (Walz et al., 1998). An approximately 300 kDa modulator complex has been shown to stimulate the attachment of regulatory particles to 20S proteasomes (Demartino et al., 1996; Adams et al., 1997). The modulator complex is constructed from three distinct subunits and stimulates the attachment of regulatory particles to 20S proteasomes and singly capped 20S proteasomes (Adams et al., 1997).

1.8 - The PA28 complex

The PA28 complex or 11S regulator is a ring shaped 180 kDa activator of 20S proteasomes

(Song et al., 1997). Two main forms of the PA28 complex have been identified. PA28α and PA28β subunits associate together in the proposed hexameric ring structure $(\alpha\beta)_3$ (Song et al., 1996). This complex was suggested to be located mainly in the cytosol (Soza et al., 1997). PA28γ (Ki antigen) subunits are proposed to associate to form a hexameric $(\gamma)_6$ structure that is predominantly localised to the nucleus (Tanaka & Chiba, 1998). These three proteins are all related, displaying ~50% amino acid sequence identity (Ahn et al., 1995). The PA28α/PA28β complex has been shown to stimulate the chymotrypsin-like, trypsin-like and PGPH activities of 20S proteasomes (Kuehn & Dahlmann, 1996; Ustrell et al., 1995). Kuehn & Dahlmann (1996) reported that the level of stimulation was dependent on the amount of PA28 complex used, with a plateau being reached at 2 moles PA28 complex per mole 20S proteasome. Phosphorylation of the PA28α/PA28β complex was proposed to be required to produce the maximum stimulation of the 20S proteasome chymotrypsin-like activity (Li et al., 1996a).

PA28α is thought to predominantly combine with PA28β under physiological conditions, although X-ray crystallographic analysis of recombinant PA28α showed that the subunits can combine to form a heptameric complex (Kuehn & Dahlmann, 1996; Knowlton et al., 1997). Recombinant PA28α complexes can also stimulate the peptidase activities of 20S proteasomes (Ustrell et al., 1995). PA28β subunits do not readily combine to form homopolymeric complexes (Zhang et al., 1998). Recent studies show that particular residues in the PA28α and PA28β subunits are important for binding and activating 20S proteasomes (Song et al., 1997; Zhang et al., 1998). In particular Arg-141 to Gly-149 of PA28α are critical for 20S proteasome activation. Deletion or mutation of residues in the C-terminal region of PA28α or PA28β also influence the ability of the PA28 complexes to bind and activate 20S

proteasomes. The precise role of PA28β is presently unclear. Some researchers suggest that this subunit cannot stimulate proteasome activity when expressed in the absence of PA28α (Song et al., 1997). PA28β was suggested to influence the affinity of PA28 complexes for the 20S proteasome. Another group suggested that PA28β can activate 20S proteasomes in the absence of PA28α and both subunits are involved in binding and activating 20S proteasomes (Zhang et al., 1998). The C2 mammalian α-type subunit has been proposed to be an important contact site for PA28 complexes (Kania et al., 1996).

Binding of PA28 complexes to the ends of the 20S proteasome has not been shown to stimulate the degradation of native, denatured or ubiquitinated proteins (Li et al., 1996a; Tanaka & Chiba, 1998). The PA28-20S proteasome complex has been implicated in the generation of antigenic peptides for presentation on MHC class I molecules (Groettrup et al., 1996b). Expression of both the PA28α and PA28β subunits is induced by IFN-γ (Li et al., 1996a). In one report recombinant PA28α was expressed in fibroblast cells, expressing the murine cytomegalovirus pp89 protein. The overexpression of PA28α lead to a marked enhancement of recognition by pp89-specific cytotoxic T-cells. Similar results were observed when control and PA28α overexpressing cells were infected with a strain of influenza and then analysed using an influenza nucleoprotein specific cytotoxic T-cell line (Groettrup et al., 1996b). These results could be explained by PA28-20S proteasomes functionally combining with 26S proteasomes, to cleave 1-2 amino acids from the 9-10 amino acid peptides produced by the 26S proteasome. The addition of PA28 complexes to 20S proteasomes was shown in a separate study to increase the diversity of peptides derived from synthetic 25 amino acid substrates (Groettrup et al., 1995). Cleavage of the peptides by PA28-20S proteasomes may increase the production of peptides that end with hydrophobic or basic residues. These peptide

are selected for expression on MHC class I molecules (Gaczynska et al., 1993).

Co-immunoprecipitation experiments have recently suggested that singly capped 20S proteasomes could bind to PA28 to form a hybrid proteasome (Hendil et al., 1998). Hybrid proteasomes might therefore be a second mechanism by which PA28 complexes increase the diversity of antigens and the level of cytotoxic T-cell recognition.

1.9 - The 26S proteasome is responsible for degrading a wide variety of proteins

The 26S proteasome has been implicated in the degradation of a large and expanding number of proteins, involved in a wide variety of cellular processes (Hilt & Wolf, 1996; Tanaka & Chiba, 1998). In this section I will briefly describe the role of proteasomes in selected areas of cell metabolism. The 26S proteasome is thought to be responsible for the degradation of most short-lived, long-lived and abnormal proteins in cells (Rock et al., 1994; Mitch & Goldberg, 1996). 26S proteasomes degrade various regulators of cell cycle progression e.g. B-type and G1 cyclins. This topic has recently been reviewed by Tanaka & Chiba (1998). Immune and inflammatory response regulators are also proposed to be degraded by the ubiquitin-proteasome pathway. For example IκB proteins that inhibits NF-κB p50-p65 complexes, are ubiquitinated and degraded by the 26S proteasome (Alkalay et al., 1995; Maggirwar et al., 1995; Whiteside et al., 1995). Phosphorylation of Ser-32 and Ser-36 was proposed to induce the ubiquitination and degradation of IκBα (Baldi et al. 1996). Recent studies have suggested that m-calpain also catalyses the degradation of IκBα in human hepatocytes (Han et al., 1999).

Proteins that fail to correctly fold in the endoplasmic reticulum (ER) are proposed to be

transported out of the ER and degraded by 26S proteasomes localised to the ER membrane (Sommer & Wolf, 1997). This was shown to be the case for mutant forms of carboxypeptidase yscY (Hiller et al., 1996) and human α-1-proteinase inhibitor (Werner et al., 1996). This pathway may also account for the degradation of incorrectly folded wild-type and ΔF508 cystic fibrosis transmembrane conductance regulator proteins (Ward et al., 1995; Jensen et al., 1995; Xiong et al., 1999). The human cytomegalovirus (HCMV) exploits this degradation pathway to induce the degradation of MHC class I heavy chains (HC) (Wiertz et al., 1996a; Wiertz et al., 1996b). Both the US2 and US11 proteins produced by HCMV, bind to glycosylated HC in the ER. This association stimulates the deglycosylation and retrograde transport of HC through the Sec61 complex of the ER membrane. The deglycosylated HC is then degraded by 26S proteasomes.

Other viruses are known to exploit the ubiquitin-proteasome pathway. For example the human papilloma virus-16 E7 protein induces the immortilization of mammary epithelial cells, by stimulating the degradation of the retinoblastoma protein (Rb) (Boyer et al., 1996). The Rb protein is a tumor suppressor protein that plays a critical role in controlling the G_1 phase of the cell cycle. The human immunodeficiency virus (HIV) type 1 proteins Env and Vpu stimulate the degradation of the helper T cell protein CD4, by 26S proteasomes (Fujita et al., 1997). CD4 is a cell surface protein that assists the attachment of helper T cell receptors to MHC class II molecules, on the surface of antigen-presenting cells. Another study showed that proteasomes form part of the cellular defense mechanism against HIV. HIV infection of target cells was much more efficient in the presence of the proteasome inhibitors Cbz-LLL-al and lactacystin (Schwartz et al., 1998). Moreover in vitro assays showed that 20S proteasomes could degrade certain HIV proteins.

1.9.1 - Role of proteasome complexes in Alzheimer's disease

Alzheimer's disease is a progressive neurodegenerative disorder which is characterised by the accumulation of ubiquitin conjugates in neurofibrillary tangles (Gregori et al., 1995; Gregori et al., 1997). Extracellular senile plaques also accumulate in the neural tissue. A principal component of these structures is the 4 kDa peptide β-amyloid (Aβ). Aβ peptides exhibit a length of 39-43 amino acids (Hamazaki, 1998). Aβ1-40 is thought to be the major species that is produced, with the longer Aβ1-42 and Aβ1-43 peptides being early and major components of the senile plaques in patients suffering from Alzheimer's disease and Down's syndrome. Aβ is derived from the amyloid precursor protein (APP), an integral type 1 membrane-spanning glycoprotein (Christie et al., 1999). APP is normally cleaved in the middle of the Aβ amino acid sequence by an α-secretase. This yields a soluble polypeptide termed sAPPα. Alternatively β- and γ-secretase activities combine to produce Aβ and a different soluble APP polypeptide. The presenilin-1 protein has been proposed to activate Aβ production by activating the γ-secretase activity (Haass & Selkoe, 1998). The enzymes responsible for the α-, β- and γ-secretase activities have proved difficult to identify. Peptidyl aldehydes have been shown to inhibit the production of Aβ and are known to inhibit proteasome activities e.g. chymotrypsin-like activity (Christie et al., 1999). Other studies have suggested that 26S proteasome are localised close to senile plaques in cases of Alzheimer's disease and dementia with Lewy bodies (Fergusson et al., 1996).

1.10 - Aims of the project

At the start of the project, very few effective and relatively specific proteasome inhibitors were available. Proteasome complexes are involved in many aspects of cellular metabolism and specific inhibitors of these complexes may prove useful in elucidating a greater understanding of various diseases e.g. arthritis, cystic fibrosis and Alzheimer's disease. These studies may lead to the use of the best inhibitors as novel therapeutic agents. Discussions with SmithKline Beecham lead to the proposal that a series of peptidyl boronic acids may prove to be highly effective inhibitors of proteasome activities. I therefore chose to characterise the effects of these compounds on the peptidase activities of purified 20S proteasomes. 20S proteasomes were purified from rat liver and rat spleen tissue and the specific activities for selected synthetic peptide substrates (Suc-LLVY-AMC, AAF-AMC, Cbz-GGL-AMC and Boc-LSTR-AMC) were determined. An initial screen of the effects of the compounds on liver 20S proteasomes was carried out, using the chymotrypsin-like substrate Suc-LLVY-AMC and the trypsin-like substrate Boc-LSTR-AMC. A kinetic analysis of the mechanism of inhibition was then performed using the most effective inhibitor, Bz-Phe-boroLeu. The structure-activity relationship of the peptidyl boronic acids for the liver 20S proteasome chymotrypsin-like activity was then investigated by determining the Ki values for a range of peptidyl boronic acids. Comparisons were made to the effects on the liver 20S proteasome trypsin-like activity, to investigate whether the order of potency was maintained for other 20S proteasome activities. The effect of peptidyl boronic acids on the spleen 20S proteasome was subsequently investigated. Spleen 20S proteasomes are constructed predominantly with IFN-γ inducible catalytic β-subunits, whereas liver 20S proteasomes contain a mixture of constitutively expressed and IFN-γ inducible catalytic β-subunits. Any difference in effects might suggest possible preferential inhibition of subpopulations of proteasomes. The peptidyl boronic acid

inhibitors were also of interest because they are cell permeable. Studies were therefore carried out to investigate whether proteasome complexes are inhibited by these compounds in cultured cells. The slow release of bound inhibitor allowed direct demonstration that proteasomes are indeed a cellular target for several of the boronic acid inhibitors. Parallel studies were carried out at SmithKline Beecham to investigate the possible role of proteasomes in the generation of β-amyloid.

Chapter 2 - Materials and Methods

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2.1 - Materials

Rats (Wistar) were obtained from B & K Universal Limited (Hull, U.K). Boc-LSTR-7-amido-4-methylcoumarin (AMC) and Succinyl (Suc)-LLVY-AMC were purchased from the Peptide Institute (Osaka, Japan). AAF-AMC, Cbz-LLE-NAP, ATP, proteinA-agarose, bovine serum albumin and acetonitrile were obtained from Sigma Chemical Co. (Poole, U.K.). Cbz-GGL-AMC was obtained from Bachem (Bubendorf, Switzerland). Solutions for cell culture were obtained from Gibco BRL (Paisley, U.K.) or Sigma Chemical Co. (Poole, U.K.). Peptidyl boronic acids were synthesized by chemists at SmithKline Beecham using the general method of Kettner & Shenvi (1984) and Matteson & Sadhu (1981). [4-^{3}H] Phenacetyl-(S)-Leu-(S)-Leu-(R,S)-boroLeu was prepared by palladium-catalysed tritiodehalogenation of parabromophenylacetyl-(S)-Leu-(S)-Leu-(R,S)-boroLeu pinacol ester. The pinacol ester group was removed using $NaIO_4$/acetone/ammonium acetate buffer to give the boronic acid. The resulting compound was 97.3% radiochemical purity by HPLC, 6.4 Ci/mmol specific activity. Cbz-LLL-vinyl sulphone was a gift from Dr Hidde Ploegh of the Dept. of Pathology, Harvard Medical School, U.S.A. The protein assay reagent for use in Bradford protein determinations was purchased from Bio-Rad (Hemel Hempstead, U.K.). Ultrapure protogel was obtained from National Diagnostics (Atlanta, U.S.A.). Sodium dodecyl sulphate (SDS) was purchased from United States Biochemicals (Cleveland, U.S.A.). Collodion bags (75 kDa cut-off) were obtained from Schleicher & Schüll (Dassel, Germany). MCP20 monoclonal anti-proteasome antibody was a gift from Dr Klavs Hendil of the August Krogh Institute, Copenhagen, Denmark. The polyclonal anti-(rat liver proteasome) antibody Ab186 was raised in rabbits as described previously using dinitrophenol-modified proteasomes (Mason et al.

1996). Optiphase HiSafe 2 liquid scintillation cocktail was obtained from Fisons Chemicals (Loughborough, U.K.).

2.2 - Protein determination by the Bradford Method

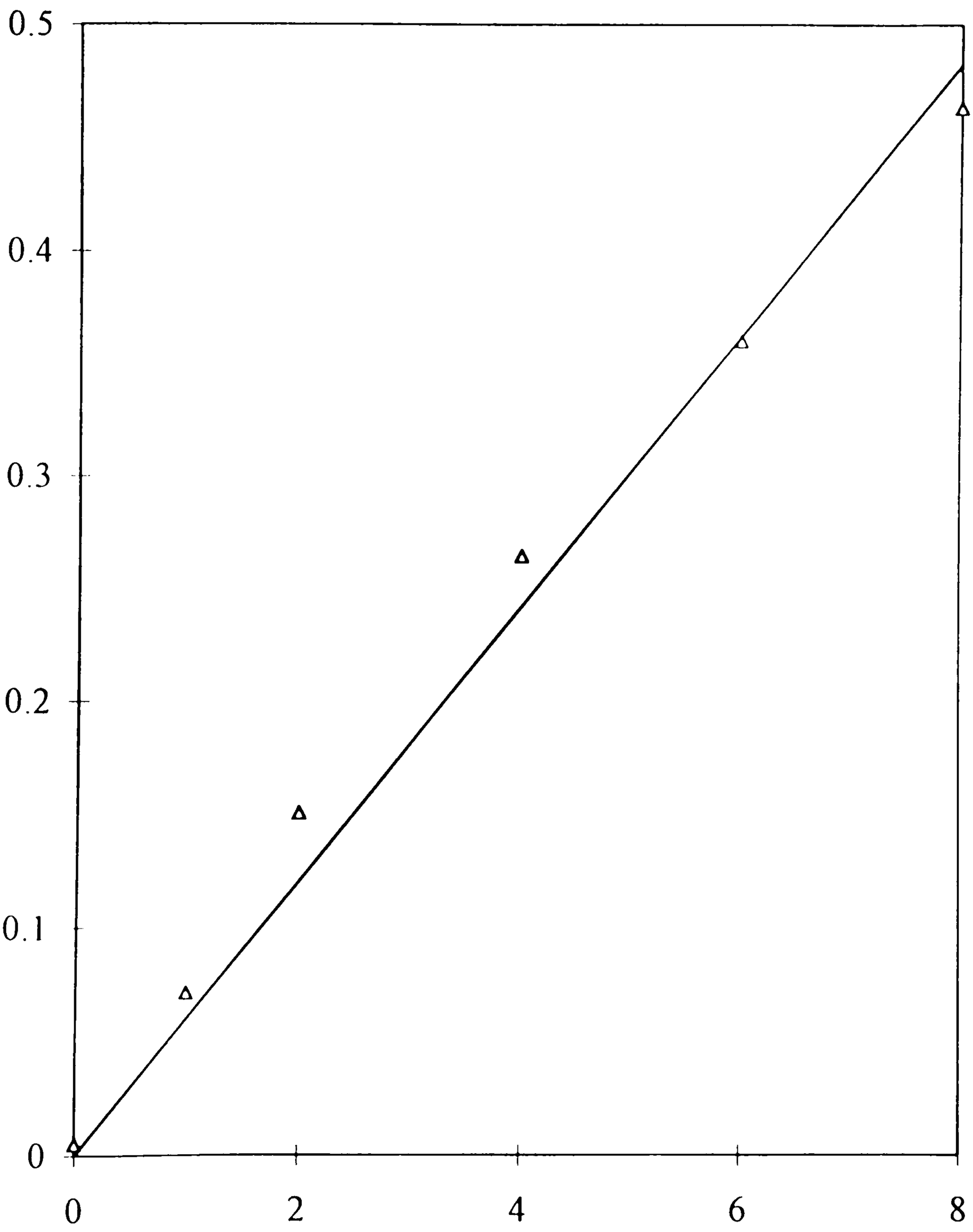
The method was essentially as described in (Bradford, 1976). Bovine serum albumin (BSA) standards of 0-8 μg were set up in duplicate in a total volume of 0.1 ml. The BSA standards were diluted with Super Q water or appropriate buffer e.g. 50 mM Hepes/KOH, pH 7.5. Samples with an estimated protein content of 1-6 μg were diluted to 0.1 ml with Super Q water or appropriate buffer. The protein assay reagent from Bio-Rad was diluted 1:5 with Super Q water. 1 ml of this diluted reagent was vortexed with each sample before incubation at room temperature for 10 minutes. The Absorbance (595 nm) of the standards and samples was then read using a 1 ml plastic curvette and a Pharmacia Ultraspec III spectrophotometer. These samples were read against the 0 μg BSA reagent blank. A typical standard curve is shown in Figure 2.1.

2.3 - SDS-PAGE and Western blotting

SDS-PAGE was conducted by the method of Laemmli (1970). Mini-gels were produced using the Bio-Rad Mini-protean II apparatus. A 15% separating gel was produced using the following protocol:

Figure 2.1 - Standard curve for the determination of protein content by the Bradford method

Samples of bovine serum albumin (0-8 μg) were processed in duplicate according to the Bradford method for protein content determination as described in section 2.2. The figure shows a typical set of results.



a) 6.30 ml of Ultrapure Protogel

b) 1.55 ml of 3 M Tris/HCl, pH 8.9

c) 4.53 ml of filtered H_2O

d) 0.125 ml of 10% SDS

e) 8 µl of TEMED (N, N, N', N'-Tetramethylethylenediamine)

f) 50 µl of 10% ammonium persulphate

A 4% stacking gel was prepared as follows:

a) 0.5 ml of Ultrapure Protogel

b) 0.276 ml of 0.5 M Tris/HCl, pH 6.8

c) 4.104 ml of filtered water

d) 50 µl of 10% SDS

e) 6 µl of TEMED

f) 20 µl of 10% ammonium persulphate

The protein samples were prepared for electrophoresis by the addition of 24 mM Tris/HCl, pH 6.8, 1% (w/v) SDS, 7.6% glycerol, 0.02% (w/v) bromophenol blue and 10% 2-mercaptoethanol. Electrophoresis was conducted at 100 or 110V for approx. 90 minutes. The gels were either stained with Coomassie stain solution or electrophoretically transferred to nitrocellulose. Gels were incubated with Coomassie stain solution (3 mM Coomassie Brilliant blue R-250, 45% ethanol, 9% glacial acetic acid) for at least 30 minutes at room temperature. Gels were destained using a solution of 40% methanol and 10% glacial acetic acid in Super Q water. Electrophoretic transfer was conducted onto nitrocellulose blotting membranes (Sartorius, Göttingen, Germany) as described previously (Towbin et al., 1979). The transfer was performed using either a Bio-Rad Trans-blot SD semi-dry transfer cell or a Bio-Rad Trans-blot cell.

2.4 - Purification of the 20S proteasome from rat liver and rat spleen

20S proteasomes were purified from rat liver as described previously (Rivett et al., 1994). This protocol was also used to purify 20S proteasomes from rat spleen tissue. Small (50-100 µl) aliquots of sample were taken at the end of each purification stage. When producing a purification table, the protein content of each sample was determined by the Bradford method as described in section 2.2. Suitable amounts of each sample were then assayed against 50 µM Suc-LLVY-AMC or 50 µM AAF-AMC. These assays were conducted according to the basic protocol of section 2.5. Greater amounts e.g. 60 µg of the crude extract or ammonium sulphate fractionation samples were used per assay tube, compared to the later samples e.g. MonoQ 10/10 column or Superose 6 column samples where 1-4 µg of protein was assayed. A fluorescence standard curve for 7-amino-4-methylcoumarin (AMC) was produced as described in section 2.5.1. The equation for this curve was used to calculate total and specific peptidase activities for the six samples from the preparation. The purity of the 20S proteasome preparation was assessed by SDS-PAGE as described in section 2.3.

2.5 - Proteasome assays

20S proteasomes were assayed against peptide substrates as described in (Rivett et al., 1994). Assays were conducted in 200 µl of 50 mM Hepes buffer/KOH, pH 7.5. The chymotrypsin-like activity was assayed against either Suc-LLVY-AMC, AAF-AMC or Cbz-GGL-AMC. These substrates were used at concentrations between 10 and 50 µM. The trypsin-like activity was assayed against 40 or 50 µM Boc-LSTR-AMC. Previous studies suggested that the cleavage of the PGPH substrate Cbz-LLE-NAP was catalysed by two components. The

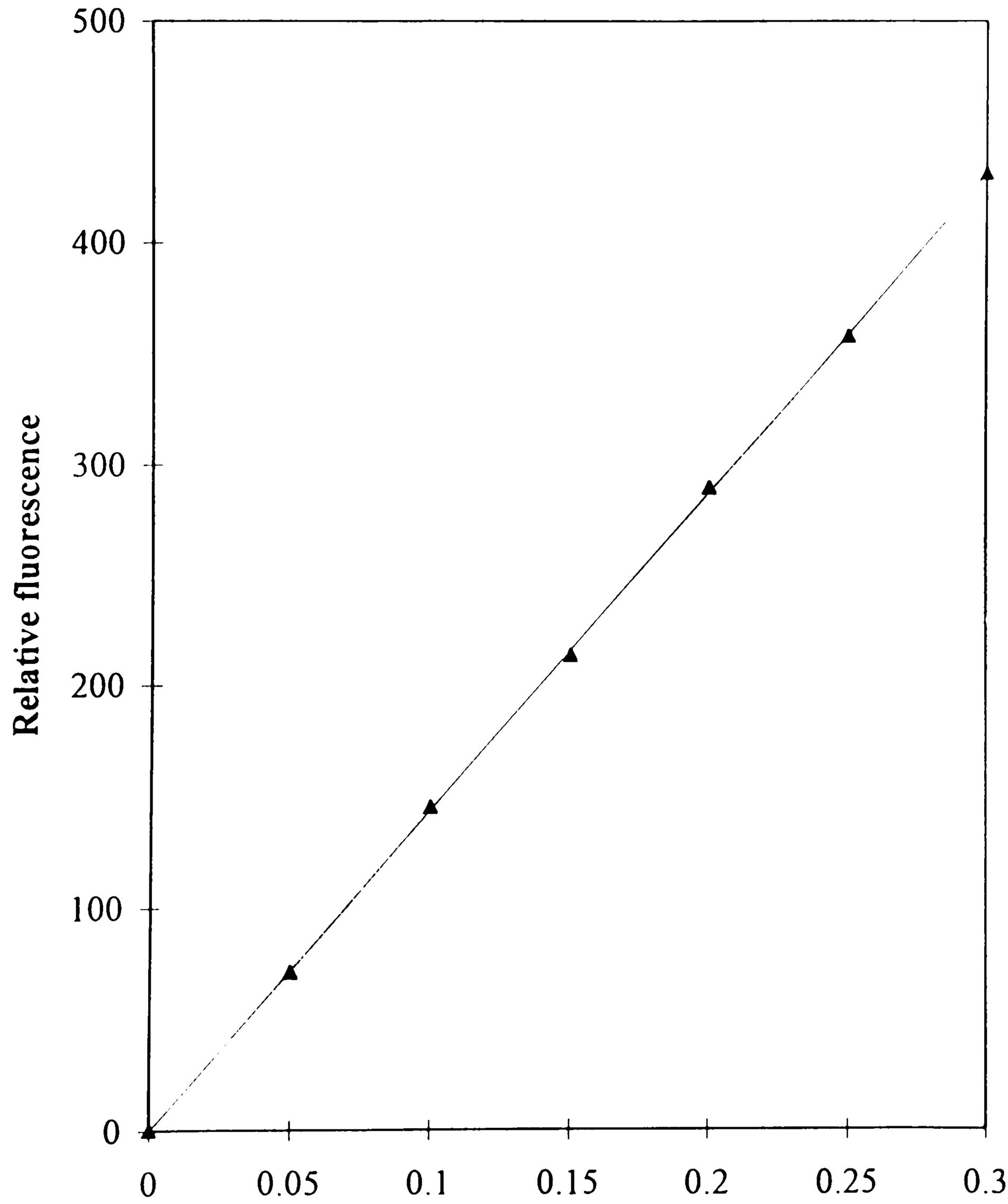
higher-affinity site obeyed Michaelis-Menten kinetics between 0-0.1 mM Cbz-LLE-NAP. The velocity increased sigmoidally with substrate concentration above 0.1 mM. These two activities were termed LLE1 and LLE2 (Djaballah & Rivett, 1992). The LLE1 activity was assayed using 0.1 mM Cbz-LLE-NAP. 20S proteasome (1 or 2 μg) were used to start the assays that were run for 15-30 minutes at 37°C. Assays for substrates with the AMC leaving group were stopped by the addition of 0.1 ml stop mix (75 mM sodium acetate trihydrate & 175 mM glacial acetic acid) and 2 ml of Super Q water. The fluorescence (excitation: 370 nm; emission: 430 nm) of the samples was measured with a Perkin-Elmer LS-3B fluorescence spectrometer. Assays with the Cbz-LLE-NAP substrate were stopped with 0.3 ml of ethanol and 2 ml of Super Q water. The fluorescence of these samples was measured with an excitation wavelength of 333 nm and an emission wavelength of 450 nm (Dahlmann et al., 1985). The fluorescence of both types of cleaved leaving group was measured against a suitable substrate blank.

2.5.1 - Production of a fluorescence standard curve for 7-amino-4-methylcoumarin (AMC)

Assay tubes were set up in duplicate with 200 μl of 50 mM Hepes buffer/KOH, pH 7.5 containing selected quantities of AMC. Stop mix (0.1 ml) and 2 ml of Super Q water was added to each assay tube. The fluorescence of the samples was read as described in section 2.5. The 0 nmol samples were used as the reagent blank. Microsoft Excel was used to plot the relative fluorescence against AMC content and determine the equation for the straight line. Figure 2.2 shows a typical standard curve determined using 0-0.3 nmols AMC. The correlation coefficient of the standard curves was always greater than 0.99.

Figure 2.2 - Standard curve for the fluorescence of 7-amino-4-methylcoumarin (AMC)

Fluorescence standard curves for 7-amino-4-methylcoumarin were determined as described in section 2.5.1. The figure shows a typical set of results using 0-0.3 nmol AMC.



2.5.2 - Determination of the Km for Suc-LLVY-AMC hydrolysis by liver 20S or 26S proteasomes and spleen 20S proteasomes

Liver 26S proteasome, liver 20S proteasome or spleen 20S proteasome (1 μg) was incubated with selected concentrations of Suc-LLVY-AMC, in 200 μl of 50 mM Hepes buffer/KOH, pH 7.5. The assays were run for 15 minutes at 37°C before stopping the reaction and measuring the fluorescence as described in section 2.5. The results were fitted to the Michaelis-Menten equation to determine the Km value. Curve fitting was performed using the following file created in the Scientist 2.0 package (MicroMath, Utah, U.S.A):

```
// MicroMath Scientist Model File
IndVars: S
DepVars: V
Params: Vmax, Km

V=Vmax*S/(Km+S)
```

2.5.3 - Time course for inhibition of the 20S proteasome LLVY activity by Bz-Phe-boroLeu

Liver or spleen 20S proteasome (1 μg) was incubated in the absence or presence of Bz-Phe-boroLeu (compound 1), in 200 μl of 50 mM Hepes buffer/KOH, pH 7.5. Suc-LLVY-AMC (40 μM) was present in the assay tubes from the beginning of the assay. The assays were run for selected time periods between 0 and 20 minutes at 37°C. The assays were stopped and fluorescence measurements were taken as described in section 2.5.

2.5.4 - Effect of peptidyl boronic acids on 20S proteasome peptidase activities

Liver or spleen 20S proteasome (1 μg) was incubated with selected concentrations of peptidyl boronic acid in 180 μl of 50 mM Hepes buffer/KOH, pH 7.5. This incubation was conducted for 15 minutes at 25°C. Substrate e.g. 20 μl of 400 μM Suc-LLVY-AMC, AAF-AMC, Cbz-GGL-AMC or Boc-LSTR-AMC in 50 mM Hepes buffer/KOH, pH 7.5 was added and the assays were run for 15 minutes at 37°C. For assays using 100 μM Cbz-LLE-AMC as substrate, the 20S proteasome was incubated with selected concentrations of peptidyl boronic acid in 150 μl of 50 mM Hepes buffer/KOH, pH 7.5. 50 μl of 400 μM Cbz-LLE-AMC was added and the assays were run for 15 minutes at 37°C. The assays were stopped and fluorescence measurements were taken as described in section 2.5.

2.6 - Kinetic analysis

Ki values for inhibition of the liver or spleen 20S proteasome LLVY activity were initially determined using the Henderson equation for tight-binding competitive inhibitors (Henderson, 1972).

$$I_t/(1-v_i/v_0)=E_t + K_i ((S_t+K_m)/K_m)v_0/v_i$$

where I_t = total inhibitor concentration, v_i = inhibited velocity, v_0 = uninhibited velocity, E_t = total enzyme concentration, K_i = dissociation constant for the binding of inhibitor to enzyme, S_t = total substrate concentration and K_m = the dissociation constant for the binding of substrate to enzyme. The data analysis was conducted using the Scientist 2.0 package. The

following Scientist file was written by Prof. Rob John of the University of Cardiff to determine the Ki from sets of v_i/v_0 (v) against I_t data:

```
// MicroMath Scientist Model File
IndVars: I
DepVars: v
Params: Ki
E=0.007
Km=129.6
S=40.0
a=E
b=-(I+E+Ki/Km*(Km+S))
c=I
x=(-b-sqrt(b^2-4*a*c))/(2*a)
v=(1-x)
```

The values of E and S were altered for experiments using 2 μg (0.014 μM) 20S proteasome or substrate concentrations of 10 or 20 μM Suc-LLVY-AMC. Similar adjustments were made when fitting various data sets to other models.

Two further models were closely examined to see if they produced a better fit to the observed data. The first of these assumed that the inhibition was occurring by a non-competitive mechanism, with the putative EI complex allowing a low rate of substrate velocity. Prof. Rob John wrote the following Scientist model file for this type of inhibition:

```
// MicroMath Scientist Model File
IndVars: I
DepVars: v
Params: Ki, r
E=0.007

a=1
b=-(I+E+Ki)
c=E*I
x=(-b-sqrt(b^2-4*a*c))/(2*a)
m= x/E
n=1-x/E
v= r*m+n
```

r = ratio of the k_{cat} values for the EIS and ES complexes. Value will be less than 1 because EIS undergoes hydrolysis to EI + P much less readily than ES hydrolyses to E + P.

The third model file written by Prof. Rob John assumed that the inhibition was occurring via a partially competitive mechanism. In this mechanism, product can be produced by ES and EIS complexes. The substrate is assumed to combine more readily with E than EI, hence the use of Km and Kmi values. Because the Ki values for the peptidyl boronic acids are very much lower than the Km for Suc-LLVY-AMC, only a single Ki value was employed in the model. Therefore this type of inhibition can be described as tight-binding partially competitive inhibition:

```
// MicroMath Scientist Model file
IndVars: I
DepVars: v
Params: Ki, Kmi, r
E=0.007
S=40.0
Km=129.6

a=(Km/S+1)*(S/Kmi+1)
Vm=100*(Km/S+1)
b=Ki/Km*(Km/S+1)*S+(S/Kmi+1)*(I-E)
c=-Ki/Km*E*S
x=(-b+sqrt(b^2-4*a*c))/(2*a)
y=I/(Kmi/Km*Ki/x +1+Kmi/S)
v=Vm*x/E+r*Vm*y/E
```

Kmi = the dissociation constant for the binding of substrate to the EI complex, r = the difference in catalytic activity of the EIS and ES complexes.

A fourth subpopulation model was also considered. This model assumed that a subpopulation of 20S proteasomes exhibited an LLVY activity that could not be inhibited by the peptidyl boronic acids. The v_i/v_0 data for each experiment was processed with following equation:

Corrected $v_i/v_0 = [(v_i/v_0) - b]/c$

Where b = the fraction of activity remaining at highest inhibitor concentration e.g. 500 nM and c = factor required to express the v_i/v_0 values as a fraction of the LLVY activity that can be inhibited. For example if v_i/v_0 at the highest inhibitor concentration is 0.14, then b = 0.14 and c = 0.86.

The corrected v_i/v_0 (v) and I values were then fitted to the competitive Henderson equation using Scientist 2.0.

2.7 - Investigating the reversibility of peptidyl boronic acid binding to liver 20S proteasomes

Liver 20S proteasome (38 μg at 0.02 μg/μl or 21.5 μg at 0.05 μg/μl) was incubated in the absence or presence of peptidyl boronic acid in 50 mM Hepes buffer/KOH, pH 7.5. The amount of DMSO added with the peptidyl boronic acid did not exceed 0.1%. The incubation was conducted for 15 or 20 minutes at 25°C. Aliquots of 20S proteasome (2 μg) were assayed against either 40 μM Suc-LLVY-AMC, AAF-AMC, Boc-LSTR-AMC or 100 μM Cbz-LLE-NAP. Assays were conducted as described in section 2.5. The remaining portions (1.1 or 0.35 ml) of 20S proteasome were dialysed separately against 400-600 or 200 ml volumes of 50 mM Hepes buffer/KOH, pH 7.5. Dialysis was conducted using collodion bags (75 kDa cut-off; Schleicher & Schüll) at 4°C overnight. The dialysed samples were assayed against the four substrates as before.

2.8 - Cell culture

L-132 human embryonic lung cells were grown in Dulbecco's modified essential medium (DMEM) supplemented with 10% (v/v) new-born bovine serum and penicillin/ streptomycin (50 IU/ml and 50 μg/ml, respectively) in a humidified atmosphere of 5% (v/v) CO_2/air (Mason et al., 1996). IMR-32 human neuroblastoma cells were cultured to 80% confluency in Dulbecco's modified Eagle's medium/F12 containing 10% (v/v) foetal calf serum, 2 mM glutamine and 50 μg/ml penicillin/streptomycin (Christie et al., 1999). IMR-32 APP-751 murine tetraploid neuronal cells were cultured to 80% confluency in a complex medium containing 60% (v/v) DMEM, 30% (v/v) Ham F-12, 5% (v/v) α-MEM, 1% (v/v) foetal calf

serum and 4% (v/v) new-born calf serum. The medium also contained 6 g/L glucose, 200 mM L-glutamine, 15 mM Hepes buffer, 1 mM dibutyryl cAMP and 2.5 μM 5-bromo-deoxyuridine. The pH of the medium was adjusted to 7.4 with 1 M NaOH (Neill et al., 1994).

2.9 - Inhibition of 20S and 26S proteasomes in cultured cells

Growth medium was removed from subconfluent L-132 cells and replaced with fresh medium containing DMSO (1%), selected concentrations of peptidyl boronic acid or Cbz-LLL-vinyl sulphone in DMSO. For experiments with IMR-32 and IMR-32 APP-751 cells the growth medium was removed and replaced with serum-free growth medium containing DMSO (1%) or selected concentrations of 2-pyrazinylcarbonyl-Phe-boroLeu. Flasks were incubated at 37°C for 2 or 24 hours before washing the cells with 3×10 ml of PBS. The cells were lysed using two different protocols designed to favour the recovery of 20S or 26S proteasomes. In the first protocol the cells were lysed with 1 ml of RIPA buffer (50 mM Tris/HCl, pH 8.0, 150 mM NaCl, 0.5% sodium deoxycholate, 1% (v/v) Nonidet P-40, 0.1% SDS) as described previously (Mason et al., 1996). RIPA buffer causes the dissociation of 26S proteasomes into 20S proteasomes and 19S regulatory complexes. Alternatively, L-132 cells were released from the flask through incubation with 1 ml of a trypsin-versene solution (1:10; GIBCO BRL) for 10 minutes at 37°C. PBS (5 ml) was added to each flask before resuspending the cells. The IMR-32 and IMR-32 APP-751 cells were scraped from the surface of the flask into the 5 ml of PBS. The cell suspensions were transferred to separate tubes and the cells were pelleted by centrifugation at 1000×g for 5 minutes. The cells were then lysed in 0.5 ml of 20 mM Tris buffer/HCl, pH 7.5 containing 5 mM ATP, 10% glycerol and 0.2% Nonidet P-40 (Mason et al., 1998). ATP and glycerol were added to the lysis buffer to maintain the structure of the

26S proteasome during cell lysis. The lysed cells were diluted 1:10 with lysis buffer containing no Nonidet P-40. The protein content of the cell lysates was determined using the Lowry method (Lowry et al., 1951). The protein in 0.2 ml of the 20S proteasome samples and 0.5 ml of the 26S proteasome samples was precipitated by the addition of 0.02% sodium deoxycholate and 8% trichloroacetic acid at 0°C. The protein was pelleted by centrifugation at 20,000×g for 15 minutes at 4°C. After discarding the supernatant, the pellets were washed with 1 ml of ice-cold 90% acetone. These samples were centrifuged as above for 10 minutes and the supernatants were discarded. The protein pellets were air-dried and then dissolved in 0.5 ml of 0.5 M NaOH. Two, 0.2 ml aliquots of each sample were used in the determination. The absorbance (750 nm) was measured using 3 ml plastic cuvettes and a Pharmacia Ultraspec III spectrophotometer.

Immunoprecipitations were carried out for the two sets of samples using the same amount of protein. MCP20 or Ab186 antibody (20 µl) and a 50% suspension of protein A-agarose in PBS (150 µl) were added to the samples. The tubes were tumbled end-over-end at 4°C for 16 hours. The 20S proteasome immunoprecipitates were washed with 3 × 1 ml of Net buffer (50 mM Tris/HCl, pH 7.0, 150 mM NaCl, 5 mM EDTA, 0.5% sodium deoxycholate, 0.1% bovine serum albumin, 0.1% SDS and 0.5% (v/v) Nonidet P-40). The 26S proteasome immunoprecipitates were washed with 3 × 1 ml of 26S lysis buffer containing 0.02% Nonidet-P40 instead of 0.2%. The 20S and 26S proteasome immunoprecipitates were then washed with 3 × 1 ml of 50 mM Hepes buffer/KOH, pH 7.5 or 50 mM Hepes buffer/KOH, pH 7.5 containing 2 mM ATP respectively. The level of buffer above the pellets was adjusted to give a 50% suspension (~150 µl total volume). Two, 60 µl aliquots of the resuspended pellets were assayed against 50 µM Suc-LLVY-AMC. The substrate was added as 40 µl of 125 µM

Suc-LLVY-AMC in either 50 mM Hepes buffer/KOH, pH 7.5 or 50 mM Hepes buffer/KOH, pH 7.5 containing 2 mM ATP for 20S and 26S proteasomes samples respectively. These assays were run for 2 hours at 37°C. After 2 hours, 20 µl of the supernatant above the pellets was removed into a separate tube and diluted with 2.28 ml of Super Q water. The fluorescence of these samples was measured as described in section 2.5. Substrate blanks were produced by running two tubes with 60 µl aliquots of 50% protein A-agarose in 50 mM Hepes buffer/KOH, pH 7.5 or 50 mM Hepes buffer/KOH, pH 7.5 containing 2 mM ATP.

2.10 - Investigating the inhibition of proteasomes in cells

Single flasks of subconfluent human embryonic lung L-132 cells were cultured in fresh medium (5 ml) amended with either 5 µl of ethanol or 5 µl of ethanol containing 100 µM or 1 mM [^{3}H] Phenacetyl-Leu-Leu-boroLeu (radiolabelled inhibitor). The resultant [^{3}H] Phenacetyl-Leu-Leu-boroLeu concentration in the culture medium was 100 nM or 1 µM respectively. The flasks were incubated for 2 hours at 37°C. Cells were released from the flask through incubation with 1 ml of a trypsin-versene solution (1:10; GIBCO BRL) for 10 minutes at 37°C. PBS (5 ml) was added to each flask before resuspending the cells. The suspension was transferred to a separate tube and the cells were pelleted by centrifugation at 1000×g for 5 minutes. The cells were then lysed in 200 µl of 20 mM Tris buffer/HCl, pH 7.5 containing 5 mM ATP, 10% glycerol and 0.2% Nonidet P-40. Lysis was conducted by passing the suspension through a 25 gauge needle. The lysate was transferred to a separate tube and centrifuged at 20,000×g for 8 minutes. The supernatant was immediately fractionated by gel filtration using a Pharmacia Superose 6 10/30 column. The column was equilibrated with 50 ml of 20 mM Tris buffer/HCl, pH 7.5 containing 5 mM ATP, 10% glycerol and 150 mM KCl.

The lysate was resolved at a flow rate of 0.2 ml/min and 1 ml fractions were collected from time zero of the run. Aliquots of each fraction (350 μM) were mixed with 12 ml of Optiphase HiSafe 2 liquid scintillation cocktail before conducting tritium counting as described in section 2.11. Two, 150 μl aliquots of the fractions were assayed against 40 μM Suc-LLVY-AMC. The assays were conducted either in the absence or presence of 0.02% SDS for 30 minutes at 37°C. Low (0.02%) concentrations of SDS activates the LLVY activity of 20S proteasomes whilest inhibiting the same activity of 26S proteasomes. Assays were stopped and the fluorescence read as described in section 2.5.

2.11 - Radioactivity measurements

Samples for tritium counting (0.2-0.35 ml) were diluted with 8 or 12 ml of Optiphase HiSafe 2 liquid scintillation cocktail in 20 ml polyethylene scintillation vials (Packard, Meriden, U.S.A.). The samples were inverted approx. 20 times and then left in the dark for at least one hour before counting. Tritium counting was performed using a Tri-Carb 1600 TR liquid scintillation analyzer (Packard, Pangbourne, U.K.). The average count per minute (cpm) reading was determined over 5 or 10 minutes for each sample. The background cpm reading was determined with suitable control samples and then used to correct the observed cpm readings.

2.12 - HPLC separation of liver 20S proteasome subunits

Liver 20S proteasome (280 μg; 1.1 μg/μl) was dialysed against 50 mM Hepes buffer/KOH, pH 7.5 using a 75 kDa collodion bag. Dialysis was conducted at 4°C for 3 hours. Dialysed

20S proteasome (270 μg) was then mixed with 1% DMSO and 0.5% trifluoroacetic acid (TFA) and incubated at room temperature for 30 minutes. TFA (0.5%) reduced the pH of the solution to approx. 2 causing the dissociation of the complex. Acetonitrile (20%) was added to the sample and the resultant solution was centrifuged for 10 minutes at 10,000×g. A Vydac C4 reverse-phase HPLC column was connected to a Hewlett-Packard 1100 HPLC system. The column was equilibrated with 10 ml of 10% acetonitrile containing 0.1% TFA. A flow rate of 0.3 ml/min was used and the column temperature was regulated to 22°C. An aliquot (200 μg) of the centrifuged sample was loaded onto the column in 10% acetonitrile containing 0.1% TFA. The column was washed with 6 ml of this buffer. The following acetonitrile gradient was then run:

Time (minutes)	**Percent acetonitrile (%)**
0	10
10	10
20	40
200	60
205	70
210	70

The absorbance (220 nm) was measured throughout the run. The results were integrated and presented using the Hewlett Packard Chemstation A.04.02 software. Fractions (2 minute; 0.6 ml) were collected from 6 minutes into the run.

2.12.1 - HPLC separation of Cbz-Leu-Leu-boroLeu (pinacol ester) (compound 8) modified liver 20S proteasome subunits

Liver 20S proteasome (280 μg; 1.1 μg/μl) was dialysed against 50 mM Hepes buffer/KOH,

pH 7.5 using a 75 kDa collodion bag. Dialysis was conducted at 4°C for 3 hours. Dialysed 20S proteasome (270 μg; 1.6 μM) was incubated with 10 μM Cbz-Leu-Leu-boroLeu (pinacol ester) (DMSO). The remaining 10 μg of 20S proteasome was incubated with 1% DMSO as a control. Both samples were incubated at 25°C for 30 minutes. Aliquots (2 μg) of the proteasome samples were assayed with 40 μM Suc-LLVY-AMC as described in section 2.5. TFA (0.5%) was added to the remaining Cbz-Leu-Leu-boroLeu (pinacol ester) modified proteasome sample. This solution was incubated at room temperature for 30 minutes. The dissociated proteasome subunits were then resolved on the Vydac C4 HPLC column as described in section 2.12.

2.12.2 - HPLC separation of [^{3}H] Phenacetyl-Leu-Leu-boroLeu (radiolabelled inhibitor) labeled liver 20S proteasome subunits

Liver 20S proteasome (150 μg; 0.8 μM) was dialysed against 50 mM Hepes buffer/KOH, pH 7.5 using a 75 kDa collodion bag. Dialysis was conducted at 4°C for 3 hours. Dialysed proteasome (130 μg) was then incubated with 5 μM [^{3}H] Phenacetyl-Leu-Leu-boroLeu (radiolabelled inhibitor) for 30 minutes at 25°C. A control sample of dialysed proteasome (13 μg; 0.8 μM) was incubated with 0.125% ethanol for 30 minutes at 25°C. Aliquots (1 μg) of radiolabelled inhibitor treated, ethanol treated or untreated 20S proteasome were assayed with 40 μM Suc-LLVY-AMC. The assays were conducted in duplicate as described in section 2.5. A PD-10 gel filtration column (Pharmacia) was equilibrated with 25 ml of 50 mM Hepes buffer/KOH, pH 7.5. The radiolabelled 20S proteasome sample (250 μl) was run onto the column together with 2.25 ml of 50 mM Hepes buffer/KOH, pH 7.5. Five, 0.5 ml fractions were collected by applying 0.5 ml aliquots of 50 mM Hepes buffer/KOH, pH 7.5 to the top of

the column. The protein content of the fractions was determined by the Bradford method as described in section 2.2. The 20S proteasomes predominantly eluted in fractions 2 and 3 that were pooled. This 1 ml sample was prepared for injection into the C4 HPLC column in 4 aliquots of 250 µl. The proteasome subunits were dissociated by the addition of 0.5% TFA and incubation at room temperature for 30 minutes. The C4 column was equilibrated with 10% acetonitrile containing 0.1% TFA as described in section 2.12. The four samples were injected into the column sequentially, washing the sample loop with 1.2 ml of 10% acetonitrile containing 0.1% TFA between injections. The acetonitrile gradient was run as described in section 2.12. Fractions (0.6 ml) were collected from 14 minutes into the gradient. Half (0.3 ml) of each fraction was mixed with 8 ml of Optiphase HiSafe 2 liquid scintillation cocktail before conducting tritium counting as described in section 2.11.

A control experiment was also performed in which 0.2 µl of 1 mM radiolabelled inhibitor, was added to 260 µl of 50 mM Hepes buffer/KOH, pH 7.5 containing 0.5% TFA. Acetonitrile (20%) was added to the sample. The sample was injected onto the Vydac C4 column and resolved using the acetonitrile gradient as described in section 2.12. Fractions (0.6 ml) were collected from 14 minutes into the gradient. Half (0.3 ml) of each fraction was mixed with 8 ml of Optiphase HiSafe 2 liquid scintillation cocktail before conducting tritium counting as described in section 2.11.

2.13 - Rapid gel filtration of [^{3}H] Phenacetyl-Leu-Leu-boroLeu (radiolabelled inhibitor) labelled liver 20S proteasomes

Liver 20S proteasome (80 µg at 0.05 µg/µl in 50 mM Hepes buffer/KOH, pH 7.5) was

incubated with 1 μM radiolabelled inhibitor for 15 minutes at 25°C. A control 20S proteasome solution (11 μg at 0.05 μg/μl in 50 mM Hepes buffer/KOH, pH 7.5) was also incubated for 15 minutes at 25°C. Aliquots (1 μg) of the radiolabelled or control proteasome solutions were assayed against either 40 μM Suc-LLVY-AMC, AAF-AMC, Boc-LSTR-AMC or 100 μM Cbz-LLE-NAP. The assays were conducted as described in section 2.5 using a 30 minute incubation at 37°C. Two PD-10 gel filtration columns (Pharmacia) were equilibrated with 25 ml of 50 mM Hepes buffer/KOH, pH 7.5. The remaining radiolabelled proteasome sample was diluted to 2.5 ml with 50 mM Hepes buffer/KOH, pH 7.5. This sample was run onto the first column. Five, 0.5 ml fractions were collected by sequentially adding 0.5 ml aliquots of 50 mM Hepes buffer/KOH, pH 7.5 to the column. Fractions 2 and 3 were pooled and diluted to 2.5 ml with 50 mM Hepes buffer/KOH, pH 7.5. This sample was applied to the second column. Five, 0.5 ml fractions were collected as above. The Bradford method was used to determine the protein concentration of the five fractions (section 2.2). Aliquots (1 μg) of 20S proteasome were then assayed against the four peptidase substrates as above.

2.14 - Investigation of the ability to block the labelling of 20S proteasomes by [^{3}H] Phenacetyl-Leu-Leu-boroLeu (radiolabelled inhibitor)

To investigate whether an irreversible inhibitor of the chymotrypsin-like activity can block incorporation of counts from the radiolabelled peptidyl boronic acid, two Eppendorf tubes were set up containing liver 20S proteasome (27.5 μg at 0.05 μg/μl in 50 mM Hepes buffer/KOH, pH 7.5). Cbz-LLL-vinyl sulphone (10 μM) was added to tube 2 and both tubes were incubated at 37°C for 60 minutes. Radiolabelled inhibitor (1 μM) was added to both tubes that were then incubated at 25°C for 15 minutes. Four PD-10 columns were equilibrated

with 25 ml of 50 mM Hepes buffer/KOH, pH 7.5. The contents of tube 1 (550 µl) were run into the first PD-10 column followed by 1950 µl of 50 mM Hepes buffer/KOH, pH 7.5. Five 0.5 ml fractions were collected by applying 0.5 ml aliquots of 50 mM Hepes buffer/KOH, pH 7.5 to the top of the column. The first four fractions were pooled. This sample was run onto the second PD-10 column followed by 0.5 ml of 50 mM Hepes buffer/KOH, pH 7.5. Five 0.5 ml fractions were collected as before. The contents of tube 2 were also fractionated using the same method and PD-10 columns 3 and 4. The Bradford method was used to determine the protein content of the fractions from PD-10 columns 2 and 4. Aliquots (100 µl) of fractions 1-4 and 2-4 were used respectively. Two, 3 µg aliquots of 20S proteasome from either PD-10 column 2 or 4 were individually mixed with 8 ml of Optiphase HiSafe 2 liquid scintillation cocktail and subjected to radioactivity counting as described in section 2.11.

Chapter 3 - Characterisation of proteasome inhibition by peptidyl boronic acids

Chapter 3 - Characterisation of proteasome inhibition by peptidyl boronic acids

3.1 - Introduction

Peptidyl boronic acids are potent inhibitors of serine proteases including pancreatic elastase, α-lytic protease and dipeptidyl peptidase IV (Zembower et al, 1996; Kettner et al., 1988; Coutts et al., 1996). In normal substrate hydrolysis the oxygen from the hydroxyl group of the catalytic serine, attacks the electrophilic carbonyl carbon of the peptide bond. A tetrahedral transition state results with the proton from the hydroxyl group being donated to the histidine of the catalytic triad. Peptidyl boronic acids also form a tetrahedral transition state complex with the catalytic serine residue. An empty p-orbital on the boron atom readily accepts the lone pair of electrons from the catalytic oxygen atom. This complex is relatively stable with the Ki values for peptidyl boronic acid inhibition often being in the low nanomolar range. For example the Ki for inhibiting the Ac-APA-pNA cleaving activity of α-lytic protease by Boc-Ala-Pro-(L)boroVal was 0.35 nM at pH 7.5 (Kettner et al., 1988). The dipeptidyl peptidase IV catalysed hydrolysis of H_2N-Ala-Pro-4-methoxy-2-napthylamide was inhibited by a number of peptidyl boronic acids. These peptidyl boronic acids had the general structure H_2N-X_{aa}-boroPro. The best IC50 values of 11 and 15 nM were obtained using L-2-aminobutyric acid or L-Ala as X_{aa} (Coutts et al., 1996). The tight association of peptidyl boronic acids to serine proteases has been used to purify mixtures of serine proteases (Zembower et al., 1996). Affinity matrices were made by removing the Cbz groups from e.g. Cbz-Ala-Ala-boroVal. The N-terminal residues were then immobilized by reaction with Sepharose 6B. This affinity matrix was used to separate mixtures of porcine pancreatic elastase with bovine α-chymotrypsin and human neutrophil elastase with human cathepsin G (Zembower et al., 1996). Peptidyl boronic

acids often exhibit slow binding to the catalytic sites of serine proteases. Inhibition of α-lytic protease catalysed Ac-APA-pNA hydrolysis by 25 nM MeOSuc-Ala-Ala-Pro-boroVal increased to the full extent after approximately 10 minutes (Kettner et al., 1988).

Recent publications have described the potent inhibition of the 20S proteasome chymotrypsin-like activity by peptidyl boronic acids (Iqbal et al., 1996; Adams et al., 1998). The peptidyl boronic acids would presumably be reacting with particular catalytic amino-terminal threonines (Seemüller et al., 1995; Groll et al., 1997; Bogyo et al., 1998). In these recent studies only one and three peptidyl boronic acids were tested respectively. Therefore no conclusions could be drawn on the best combination of amino acids for inhibiting the chymotrypsin-like activity. One dipeptidyl boronic acid containing the N^{γ}-nitroArg-Leu combination of amino acids, did not inhibit the trypsin-like activity at 1 μM concentration (Iqbal et al., 1996). The effect of peptidyl boronic acids on other 20S proteasome activities were not investigated. Chemists at SmithKline Beecham synthesized a large number of peptidyl boronic acids for use in their experimental work. In this chapter I will describe initial experiments that characterised the effects of a number of di- and tripeptidyl boronic acids on the peptidase activities of the 20S proteasome.

3.2 - Purification of the 20S proteasome from rat liver and determination of the Km for Suc-LLVY-AMC hydrolysis

20S proteasomes were purified from rat liver as described previously (Rivett et al., 1994). Table 3.1 shows the purification table from a typical liver preparation using 230 g of rat liver. Subsequent preparations yielded up to 11 mg of 20S proteasome with similar specific activities against the Suc-LLVY-AMC and AAF-AMC substrates.

Table 3.1 - Purification of the 20S proteasome from rat liver

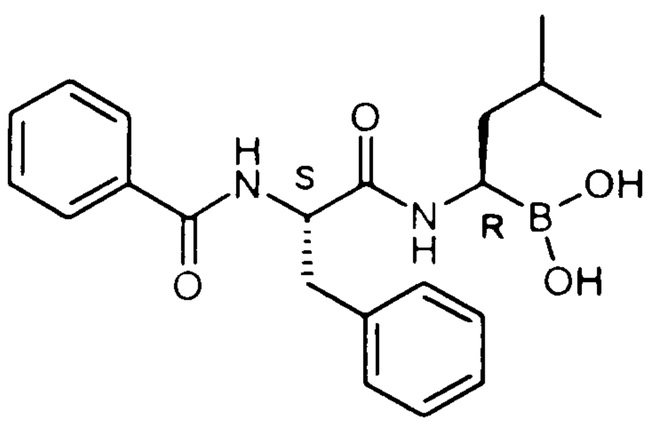
Step	Volume (ml)	Protein concn (mg/ml)	Total protein (mg)	LLVY activity		AAF activity	
				Total activity (nmol/min)	Specific activity (nmol/ min/mg protein)	Total activity (nmol/min)	Specific activity (nmol/ min/mg protein)
1) Crude extract	1220	42.1	51414	4361	0.1	83376	1.6
2) $(NH_4)_2SO_4$ fractionation	350	52.9	18500	3286	0.2	31986	1.7
3) DEAE-cellulose	570	2.5	1411	654	0.5	723	0.5
4) Mono-Q 10/10	6	13.8	83	134	1.6	303	3.7
5) Superose 6	12	0.8	9	51	5.6	42	4.6
6) Purified	6.5	0.4	3	22	7.8	24	8.7

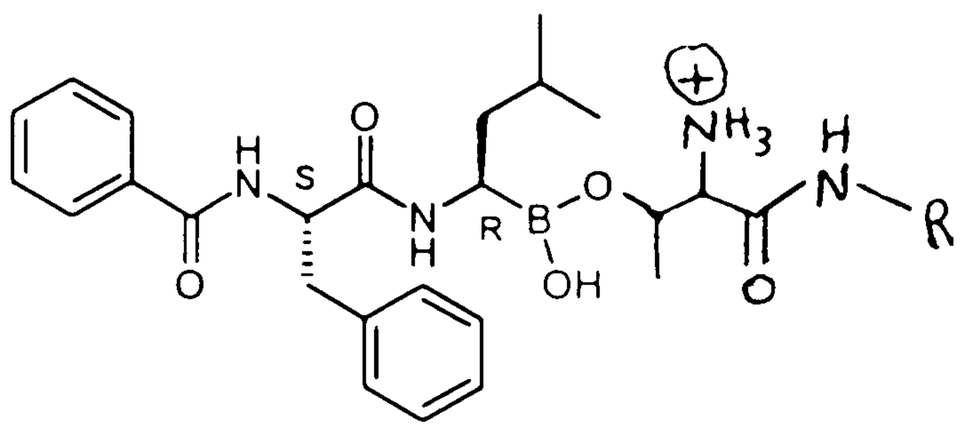
20S proteasomes were purified from 230 g of frozen rat liver as described previously (Rivett et al., 1994). Small samples were frozen after each step for subsequent protein determination (Bradford method) and enzyme assay with 50 μM Suc-LLVY-AMC (LLVY) or 50 μM AAF-AMC (AAF).

The Km for Suc-LLVY-AMC hydrolysis by liver 20S proteasomes was determined using selected concentrations of Suc-LLVY-AMC and 1 μg portions of liver 20S proteasome as described in Chapter 2. Figure 3.1 shows a typical set of data fitted to the Michaelis-Menten equation using Scientist 2.0. The rate of hydrolysis ceased to follow Michaelis behaviour above 150 μM. This was probably due to product accumulation rather than substrate depletion, as approx. 5% of the substrate was cleaved using 200 μM Suc-LLVY-AMC. The average Km value from 4 determinations was 130 ± 23 μM. The Km for the 26S proteasome LLVY activity was also determined. A typical set of data for the 26S proteasome is also shown in Figure 3.1. The Km was determined to be 95 μM (range ± 2) from two determinations. Under normal assay conditions the LLVY activity of the 26S proteasome is 5-6 times higher than the 20S proteasome. This difference can be explained partially by the lower Km value but primarily by the 4.5 times higher Vmax value. Hence the 19S regulatory groups substantially increase the rate of catalysis of this substrate, without significantly affecting its ability to bind to the appropriate catalytic sites.

3.3 - Time course for inhibition of the liver 20S proteasome LLVY activity by peptidyl boronic acids is consistent with slow binding

Figure 3.2 shows the time course for inhibition of the liver 20S proteasome LLVY activity by 10 nM Bz-Phe-boroLeu (compound 1). Below are the chemical structures of Bz-Phe-boroLeu (left) and the predicted ester bond between Bz-Phe-boroLeu and a catalytic threonine (right)





Preliminary studies had shown that a number of peptidyl boronic acids caused potent inhibition of the liver 20S proteasome LLVY activity. In these assays the peptidyl boronic acid was preincubated with 20S proteasome for 15 minutes. This allowed the peptidyl boronic acid to bind to the 20S proteasome before addition of substrate. Compound 1 was shown to be the most potent inhibitor of the 20S proteasome LLVY activity. In the time course assays 20S proteasome (1 μg) was added to assay tubes containing either substrate or substrate and compound 1. Assays were performed at 37°C for selected times. The LLVY activity in the absence of inhibitor was linear for at least 20 minutes. In the presence of 10 nM compound 1 the fully inhibited rate of reaction was only observed after approximately 10-12 minutes. This is in contrast to classical reversible inhibitors, that produce the fully inhibited reaction rate from time zero. The enzyme concentration used in the experiment was 7 nM. Therefore compound 1 can be described as a tight-binding inhibitor that inhibits the LLVY activity at concentrations comparable to the enzyme concentration (Morrison, 1982). The slow-binding behaviour could be explained by steric hinderance, slowing the passage of inhibitor to the catalytic sites. This may be a significant factor for the 20S proteasome, which has catalytic sites embedded in the centre of the complex. Also a slow conformational change may occur following inhibitor binding to the catalytic threonine(s). This conformation change would yield the fully inhibited structure.

Previous studies have shown that peptidyl boronic acids often exhibit slow-binding inhibition of serine proteases. Slow-binding inhibition is particularly common with peptidyl boronic acids that are substrate analogues (Kettner et al., 1988; Bachovchin et al., 1988). MeOSuc-Ala-Ala-Pro-boroVal was shown to be a slow-binding inhibitor of α-lytic protease (Kettner et al., 1988). The cleavage of 4 mM Ac-APA-pNA by 5 nM α-lytic protease was only fully

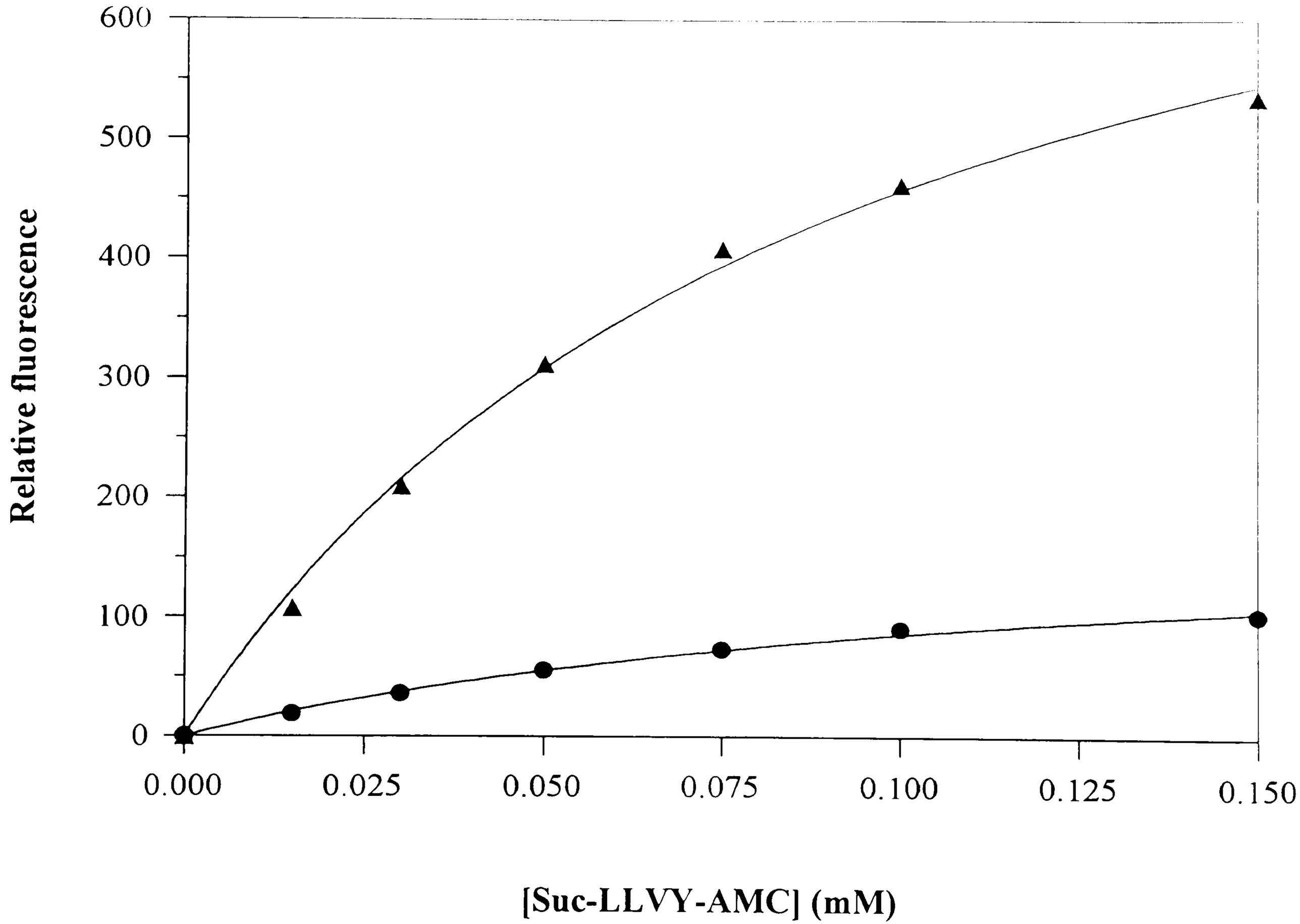
inhibited by 25 nM MeOSuc-Ala-Ala-Pro-boroVal, after 10 minutes incubation at 25°C. This slow-binding behaviour was observed even with a relatively high (4 × Ki) concentration of inhibitor. Bachovchin and coworkers (1988) used ^{15}N and ^{1}H NMR spectroscopy to investigate the binding of peptidyl boronic acids to the serine protease α-lytic protease. They suggested that a large activation energy may need to be overcome before covalent bond formation occurs. Hence the residues of the catalytic site may need to move following inhibitor binding to ease covalent bond formation. Slow binding of peptidyl aldehydes to the Cbz-GGF-pAB cleaving site of pituitary 20S proteasomes was previously observed. Micromolar concentrations of Cbz-LLF-al or Ac-LL-Nle-al needed 20-30 minutes preincubation with 20S proteasome before exerting the full inhibitory effect (Vinitsky et al., 1992). Analysis of the progress curves suggested that the peptidyl aldehydes were either binding slowly in a single step or rapidly binding to the catalytic site before undergoing a slow conformational change that exerted full inhibition of catalysis.

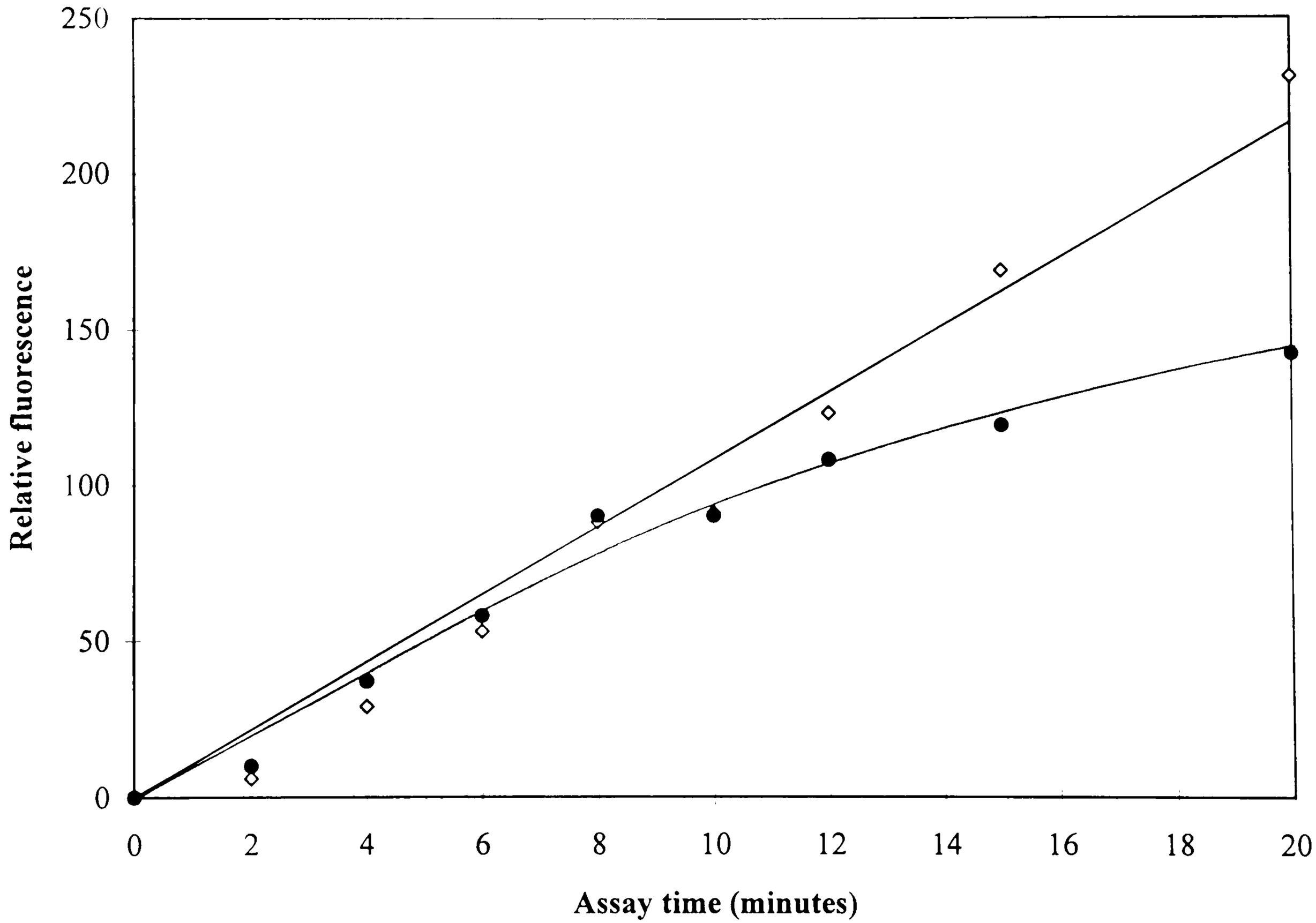
Figure 3.1 - Hydrolysis of Suc-LLVY-AMC by liver 20S and 26S proteasomes (page 71)

The 20S (circles) or 26S (triangles) proteasomes were assayed with a range of Suc-LLVY-AMC concentrations in 50 mM Hepes buffer/KOH, pH 7.5. Assays were conducted for 15 minutes at 37°C, using 1 μg of either proteasome form. Results from a typical experiment performed in duplicate are shown. Mean Km values were determined by fitting the data from separate experiments to the Michaelis-Menten equation. Values of 130 μM (S.D. ± 23, n=4) for 20S proteasomes and 95 μM (range ± 2, n=2) for 26S proteasomes were calculated.

Figure 3.2 - Time course for inhibition of the liver 20S proteasome LLVY activity by 10 nM Bz-Phe-boroLeu (compound 1) (page 72)

Liver 20S proteasomes (1 μg) were incubated at 37°C in the presence (filled circles) or absence (open diamonds) of 10 nM compound 1. Substrate (40 μM Suc-LLVY-AMC) was present from time zero and substrate hydrolysis was determined after incubation for selected times as described in Chapter 2.


600
500
400
300
200
100
0
0.000
0.025
0.050
0.075
0.100
0.125
0.150
Relative fluorescence
[Suc-LLVY-AMC] (mM)


250
200
150
100
50
0
0
2
4
6
8
10
12
14
16
18
20
Relative fluorescence
Assay time (minutes)

3.4 - Effect of Bz-Phe-boroLeu (compound 1) on selected activities of liver 20S proteasomes

Table 3.2A shows the effect of compound 1 on the LLVY, LSTR, GGL, AAF and LLE1 activities of liver 20S proteasomes. Compound 1 caused inhibition of the LLVY activity at both 20 and 80 nM concentrations. Compound 1 was much less effective against the other peptidase activities. The LLE1 activity was substantially inhibited at 80 nM compound 1, suggesting an IC50 value of approximately 100 nM. 80 nM compound 1 did not significantly inhibit the cleavage of the chymotrypsin-like substrates Cbz-GGL-AMC or AAF-AMC. Previous studies have employed peptidyl chloromethanes as inhibitors of the 20S proteasome. AAF-CH_2Cl, LLE-CH_2Cl and Cbz-LLE-CH_2Cl were all more potent inhibitors of the LLVY activity than the AAF activity. Taken together these results suggest that the chymotrypsin-like substrates AAF-AMC and Cbz-GGL-AMC are partially hydrolysed at a site distinct from the Suc-LLVY-AMC cleaving site (Reidlinger et al., 1997; Savory et al., 1993). In another study the antitumor drug Aclacinomycin A inhibited the LLVY activity of pituitary 20S proteasomes, to a greater extent than the cleavage of the chymotrypsin-like substrates Cbz-EAL-pNA or Cbz-GGL-pNA (Figueiredo-Pereira et al., 1996). The hydrolysis of Cbz-IEAL-pNA and Suc-LLVY-AMC was inhibited to a similar extent using 50 and 100 μM Aclacinomycin A.

Compound 1 (20 and 80 nM) caused a slight stimulation of the LSTR activity. This effect has been observed with other types of proteasome inhibitor e.g. 3,4-dichloroisocoumarin and certain peptidyl chloromethanes (Djaballah et al., 1992; Savory et al., 1993). Stimulation of the LSTR activity may result from inhibitor binding to non-trypsin-like sites, causing conformational changes that stimulate the trypsin-like activity.

3.4.1 - Cbz-Leu-boroLeu (80 nM) only causes significant inhibition of the liver 20S proteasome LLVY activity

Cbz-Leu-boroLeu was found to be an effective inhibitor of the liver 20S proteasome LLVY activity. Table 3.2B illustrates the effect of 20 and 80 nM Cbz-Leu-boroLeu on the LLVY, LSTR, GGL, AAF and LLE1 activities of liver 20S proteasomes. Only the LLVY activity was inhibited by 20 and 80 nM Cbz-Leu-boroLeu. Of note is the fact that compared to Bz-Phe-boroLeu (compound 1), 80 nM Cbz-Leu-boroLeu caused much less inhibition of the LLE1 activity and no stimulation of the LSTR activity. This suggests that modification of the LLE1 catalysing site(s) by compound 1 may cause the observed stimulation of the LSTR activity. Alternatively, the modification of the LLVY cleaving sites by the smaller Cbz-Leu-boroLeu compound, may not cause the conformational changes that stimulate the LSTR activity.

Table 3.2 - Inhibition of the liver 20S proteasome chymotrypsin-like activity by Bz-Phe-boroLeu (compound 1) (Panel A) and Cbz-Leu-boroLeu (Panel B)

Panel A

Substrate	Activity (% of control) 20 nM Bz-Phe-boroLeu	Activity (% of control) 80 nM Bz-Phe-boroLeu
Suc-LLVY-AMC	53	31
AAF-AMC	89	82
Cbz-GGL-AMC	93	82
Boc-LSTR-AMC	113	124
Cbz-LLE-NAP	96	61

Panel B

Substrate	Activity (% of control) 20 nM Cbz-Leu-boroLeu	Activity (% of control) 80 nM Cbz-Leu-boroLeu
Suc-LLVY-AMC	65	34
AAF-AMC	96	86
Cbz-GGL-AMC	100	90
Boc-LSTR-AMC	96	98
Cbz-LLE-NAP	97	84

Assays were performed as described in Chapter 2 using 1 μg of liver 20S proteasome. The substrate concentration was 40 μM for Suc-LLVY-AMC, AAF-AMC, Cbz-GGL-AMC and Boc-LSTR-AMC. Cbz-LLE-NAP was used at 100 μM. Values are from two experiments (Panel A) or one experiment (Panel B) performed in duplicate. These values are expressed as percent of control activity in samples containing no inhibitor.

3.5 - Modification of liver 20S proteasome catalytic sites by peptidyl boronic acids is reversible

Dialysis was employed to confirm that the effects of peptidyl boronic acids on 20S proteasome activities were reversible. In one experiment liver 20S proteasome (38 μg at 0.02 μg/μl) was incubated with either 0, 70 nM or 1 μM compound 1 in 50 mM Hepes buffer/KOH, pH 7.5. After a preincubation of 15 minutes at 25°C, 2 μg portions of the liver 20S proteasome were assayed against either 40 μM Suc-LLVY-AMC, AAF-AMC, Boc-LSTR-AMC or 100 μM Cbz-LLE-NAP. The remaining 20S proteasome solutions (1.1 ml) were dialysed separately against 50 mM Hepes buffer/KOH, pH 7.5 using 75 kDa collodion bags. The peptidase assays were then repeated with the dialysed 20S proteasome. The results of this experiment are shown in Table 3.3. Overnight dialysis only partially reversed the inhibition of the LLVY activity. Compound 1 therefore exhibited a slow off-rate from the chymotrypsin-like catalytic sites. This was the case even though the concentration of compound 1 around the 20S proteasomes had been reduced to 0.1 and 1.6 nM respectively. The inhibition of the AAF and LLE1 activities was also not readily reversed. Table 3.4 shows the results of a similar dialysis experiment using 1 μM of either compound 1, Cbz-Leu-boroNle (pinacol ester) or Cbz-Leu-Leu-boroLeu (pinacol ester). The results in Tables 3.3 and 3.4 confirm that inhibition of the LLVY, AAF and LLE1 activities by compound 1 can be slowly reversed. Sufficient time as well as changes in dialysis buffer would be required to recover full activity. Compound 1 was found to either stimulate or have no effect on the LSTR activity. This stimulation tended to increase rather than be reversed by dialysis. Stimulation of the LSTR activity may result from inhibitor binding to non-trypsin-like catalytic sites, causing conformational changes that stimulate activity at trypsin-like catalytic sites. The reason why the observed stimulation

should increase during dialysis was unclear.

Compared to compound 1, Cbz-Leu-boroNle (pinacol ester) was a less effective inhibitor of the liver 20S proteasome LLVY, AAF and LLE1 activities. In contrast to compound 1 the effects of Cbz-Leu-boroNle (pinacol ester) on these activities were all readily reversible. This illustrates the fact that the amino acid composition of a peptidyl boronic acid will affect both the potency of inhibition and rate of detachment from a particular catalytic site. The effects of 1 μM Cbz-Leu-Leu-boroLeu (pinacol ester) on the LLVY, AAF and LLE1 activities were similar to 1 μM Cbz-Leu-boroNle (pinacol ester). Cbz-Leu-Leu-boroLeu (pinacol ester) (1 μM) also caused significant inhibition of the LSTR activity. Only the small inhibition of the LLE1 activity by this inhibitor appeared to be readily reversible. Modification of both the chymotrypsin-like and trypsin-like catalytic sites was slowly reversible. Compared to Cbz-Leu-boroNle (pinacol ester), the longer Cbz-Leu-Leu-boroLeu (pinacol ester) maybe restrained more in the vicinity of the catalytic site. This could lead to the inhibitor repeatedly rebinding to the catalytic site.

3.5.1 - Photoaffinity labelling (PAL) peptidyl boronic acids are highly effective inhibitors of the 20S proteasome LLVY activity that slowly detach from the complex

Chemists from SmithKline Beecham supplied four photoaffinity labelling (PAL) peptidyl boronic acids. These peptidyl boronic acids contain a photoactivatable group e.g. benzophenone or phenyl azide that is activated by U.V. light. Activation of this group creates a reactive species that can covalently bind to nearby protein residues. All four PAL peptidyl boronic acids caused inhibition of the liver 20S proteasome LLVY activity (Table 3.5). SBFC and SBFE exhibited a similar potency to compound 1 at 1 μM concentration. The PAL peptidyl boronic acids were found to slowly detach from the LLVY hydrolysing sites of the 20S proteasomes, during overnight dialysis.

Table 3.3 - Binding of Bz-Phe-boroLeu (compound 1) to liver 20S proteasomes is not readily reversible

Peptidase activity	70 nM Bz-Phe-boroLeu		1 μM Bz-Phe-boroLeu	
	Activity (% of control) after 15 min preincubation	Activity (% of control) after subsequent dialysis	Activity (% of control) after 15 min preincubation	Activity (% of control) after subsequent dialysis
LLVY	36	79	8	36
AAF	88	88	53	63
LSTR	136	125	99	131
LLE1	N.D.	N.D.	34	33

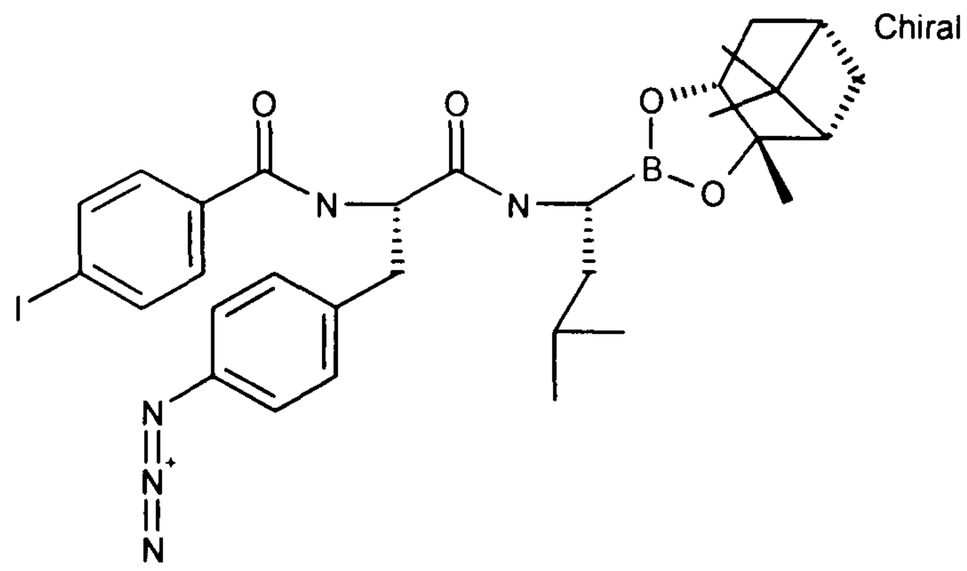
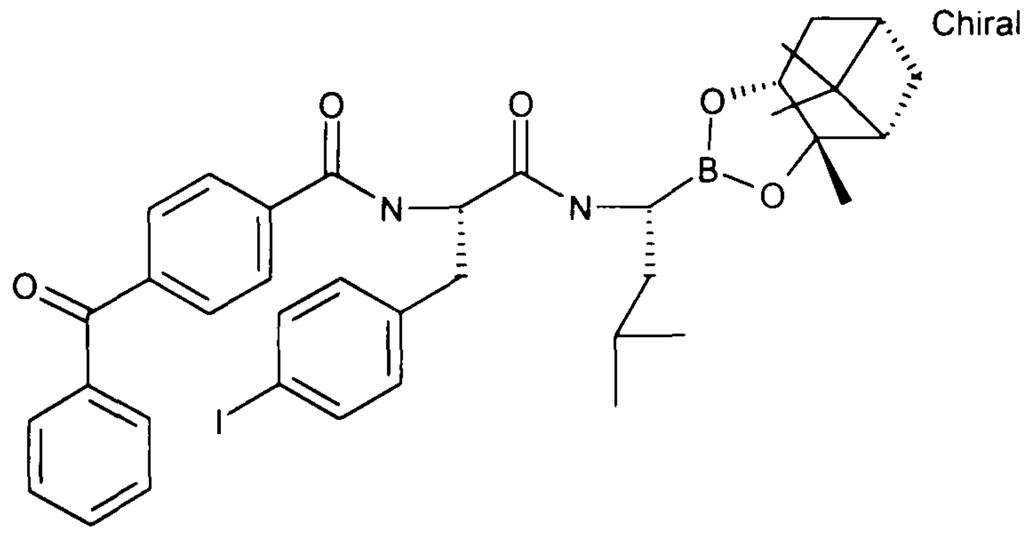
Liver 20S proteasome (38 μg at 0.02 μg/μl) was incubated with either 0, 70 nM or 1 μM compound 1 in 50 mM Hepes buffer/KOH, pH 7.5. The incubation was conducted for 15 minutes at 25°C. 20S proteasome aliquots (2 μg) were assayed against either 40 μM Suc-LLVY-AMC, AAF-AMC, Boc-LSTR-AMC or 100 μM Cbz-LLE-NAP. Assays were conducted as described in Chapter 2. The remaining portions (1.1 ml) of 20S proteasome were dialysed separately against approx. 600 ml volumes of 50 mM Hepes buffer/KOH, pH 7.5. Dialysis was conducted using 75 kDa collodion bags at 4°C overnight. The dialysed samples were assayed against the four substrates as before. Values are given as the average of a single experiment performed in duplicate. These values are expressed as percent of control activity in samples containing no inhibitor. N.D. - not determined.

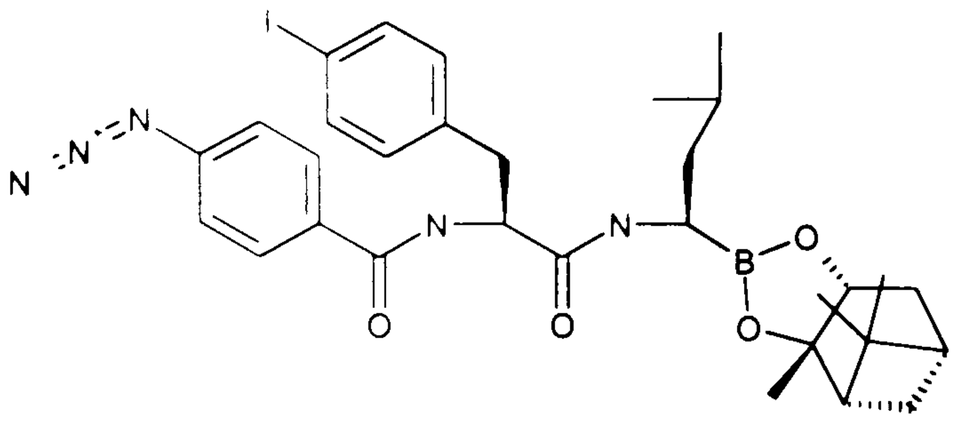
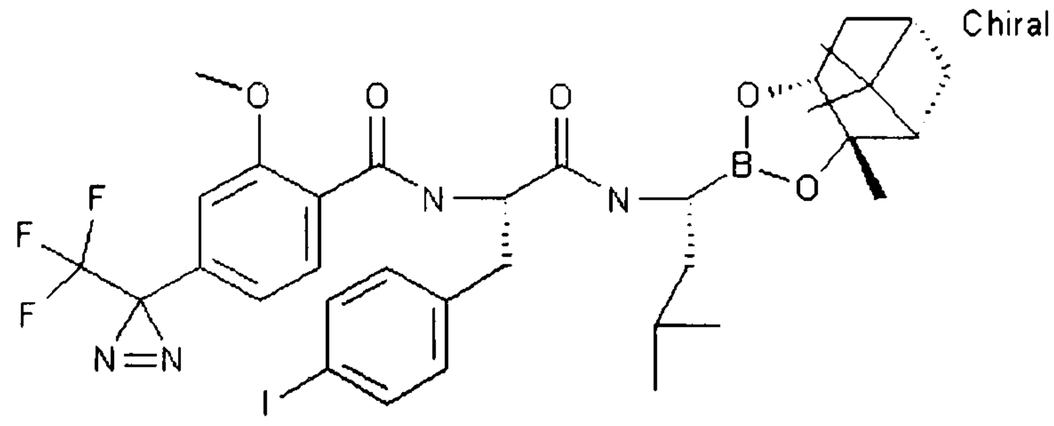
Table 3.4 - Reversibility of inhibition of 20S proteasome activities by Bz-Phe-boroLeu (compound 1), Cbz-Leu-boroNle (pinacol ester) and Cbz-Leu-Leu-boroLeu (pinacol ester)

Peptidase activity	Bz-Phe-boroLeu		Cbz-Leu-boroNle (pinacol ester)		Cbz-Leu-Leu-boroLeu (pinacol ester)	
	Activity (% of control) after 15 min preincubation	Activity (% of control) after subsequent dialysis	Activity (% of control) after 15 min preincubation	Activity (% of control) after subsequent dialysis	Activity (% of control) after 15 min preincubation	Activity (% of control) after subsequent dialysis
LLVY	6	27	16	95	7	64
AAF	38	43	76	91	79	85
LSTR	117	172	107	111	59	81
LLE1	22	69	61	97	80	94

Liver 20S proteasome (38 μg at 0.02 μg/μl) was incubated without peptidyl boronic acid or with either 1 μM Bz-Phe-boroLeu, Cbz-Leu-boroNle (pinacol ester) or Cbz-Leu-Leu-boroLeu (pinacol ester) in 50 mM Hepes buffer/KOH, pH 7.5. This incubation was conducted for 20 minutes at 25°C. Aliquots (2 μg) of 20S proteasome were assayed against 40 μM Suc-LLVY-AMC, AAF-AMC, Boc-LSTR-AMC or 100 μM Cbz-LLE-NAP. The assays were conducted as described in Chapter 2. The remaining portions (1.1 ml) of 20S proteasome were dialysed separately against approx. 400 ml volumes of 50 mM Hepes buffer/KOH, pH 7.5. Dialysis was conducted using 75 kDa collodion bags at 4°C overnight. The dialysed samples were assayed as before. Values are the result of a single experiment conducted in duplicate. These values are expressed as percent of control activity in samples containing no inhibitor

Table 3.5 - Photoaffinity labelling (PAL) peptidyl boronic acids do not readily detach from liver 20S proteasome chymotrypsin-like catalytic sites

PAL peptidyl boronic acid	LLVY activity (% of control)	
	After 20 min preincubation	After overnight dialysis
 SBFC ((4-iodo)Bz-(4-azido)benzyl-boroLeu (pinane diol ester))	9	17
 SBFD ((4-benzoyl)Bz-(4-iodo)benzyl-boroLeu (pinane diol ester))	22	40

 SBFE ((4-azido)Bz-(4-iodo)benzyl-boroLeu (pinane diol ester))	13	36
 SBFF ((2-methoxy-4-(2,2,2-trifluoro-(1,1-diazeno)ethyl))Bz-(4-iodo)benzyl-boroLeu (pinane diol ester))	23	36

Liver 20S proteasome (21.5 μg at 0.05 μg/μl) was incubated without peptidyl boronic acid or with 1 μM SBFC, SBFD, SBFE or SBFF in 50 mM Hepes buffer/KOH, pH 7.5. The incubation was conducted for 20 minutes at 25°C. 20S proteasome aliquots (2 μg) were assayed against 40 μM Suc-LLVY-AMC as described in Chapter 2. The remaining portions (0.35 ml) of 20S proteasome were dialysed separately against approx. 200 ml volumes of 50 mM Hepes buffer/KOH, pH 7.5. Dialysis was conducted using 75 kDa collodion bags at 4°C overnight. The dialysed samples were assayed against the LLVY substrate as before. Values are given as the average of two experiments performed in duplicate. These values are expressed as percent of control activity in samples containing no inhibitor.

3.6 - High concentrations of compound 1 cause near complete inhibition of the liver 20S proteasome LLVY activity

Figure 3.3 shows the effect of 0-500 nM compound 1 on the liver 20S proteasome LLVY activity. The inhibition data were initially fitted to the Henderson equation for tight-binding competitive inhibitors (Henderson, 1972). The curve generated using the competitive Henderson equation fitted the data points up to approximately 3×Ki. The observed inhibition above 3×Ki was less than predicted by the Henderson equation on each of the three occasions the experiment was performed. This was also shown to be the case for other peptidyl boronic acids. For example Figure 3.4 shows the effect of selected concentrations of Cbz-Leu-Leu-boroLeu on the 20S proteasome LLVY activity. The deviation from ideal behaviour above 2×Ki was observed in all four expts with this inhibitor. To confirm that the maximum effect of compound 1 was exerted at higher inhibitor concentrations, the experiment was repeated using preincubation times of 15 and 60 minutes. The results of this experiment are shown in Figure 3.5. Increasing the preincubation time did not increase the effectiveness of compound 1 at either low or high inhibitor concentrations. Because of the poor fit of the competitive Henderson equation to the experimental data above 2-3 Ki, other equations were used to attempt to produce a better curve fit to the whole of the data. The first model assumed that the inhibition was non-competitive i.e. inhibitor was binding to residues distinct from the principal LLVY hydrolysing sites. These residues could be associated with other catalytic sites. The model also assumed that the proteasome-inhibitor complex allowed a low level of Suc-LLVY-AMC hydrolysis. This may explain why the level of activity fails to closely approach zero at high inhibitor concentrations. The equation was written by Prof. Rob John of University of Wales at Cardiff and is shown in Chapter 2.

Figure 3.3 - Inhibition of the liver 20S proteasome LLVY activity by Bz-Phe-boroLeu (compound 1) (page 86)

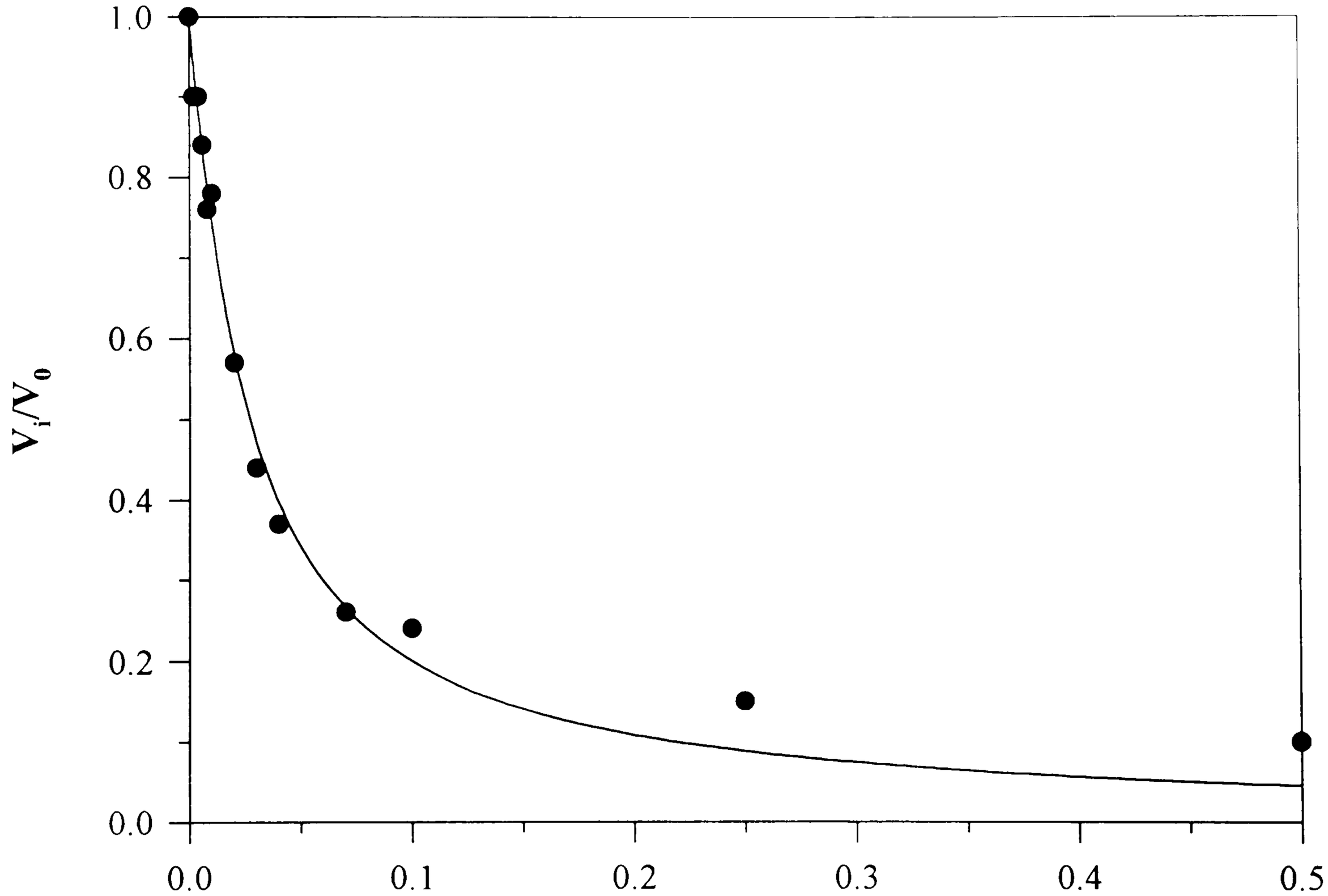
Liver 20S proteasome (1 μg) was incubated with selected concentrations of compound 1 in 50 mM Hepes buffer/KOH, pH 7.5, for 15 minutes at 25°C. Substrate (40 μM Suc-LLVY-AMC) was added and the assays were run for 15 minutes at 37°C, as described in Chapter 2. Values are from a typical experiment performed in duplicate. Activities are expressed as the fraction of control activity in samples containing no inhibitor. The competitive Henderson equation for tight-binding inhibitors was used to generate a curve fit to the data.

Figure 3.4 - Inhibition of the liver 20S proteasome LLVY activity by Cbz-Leu-Leu-boroLeu (page 87)

An experiment was performed as described in the legend to Figure 3.3 using selected concentrations of Cbz-Leu-Leu-boroLeu.

Figure 3.5 - Increasing the length of preincubation does not increase the extent of inhibition of the 20S proteasome LLVY activity by Bz-Phe-boroLeu (compound 1) (page 88)

Liver 20S proteasome (1 μg) was incubated with selected concentrations of compound 1 in 50 mM Hepes buffer/KOH, pH 7.5. The incubation was conducted for 15 (solid circles) or 60 (open triangles) minutes at 25°C. Substrate (40 μM Suc-LLVY-AMC) was added and the assays were incubated at 37°C for 15 minutes, as described in Chapter 2. Values are from one experiment performed in duplicate. Activities are expressed as percent of control activity in samples containing no inhibitor.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0.4
0.2
0.0
V_i/V_0
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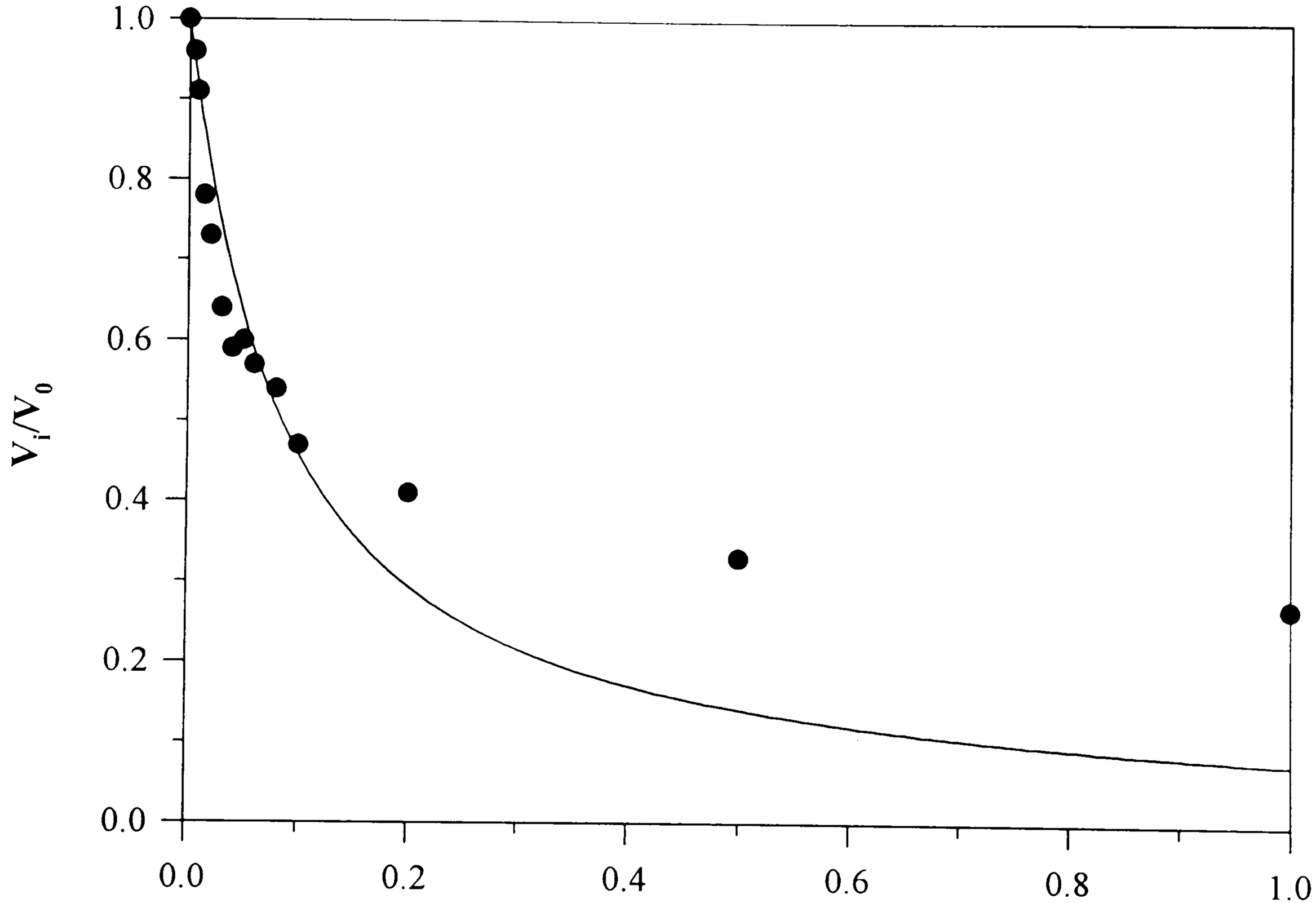
0.1
0.2
0.3
0.4
0.5
[Bz-Phe-boroLeu] (μM)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0.6
0.4
0.2
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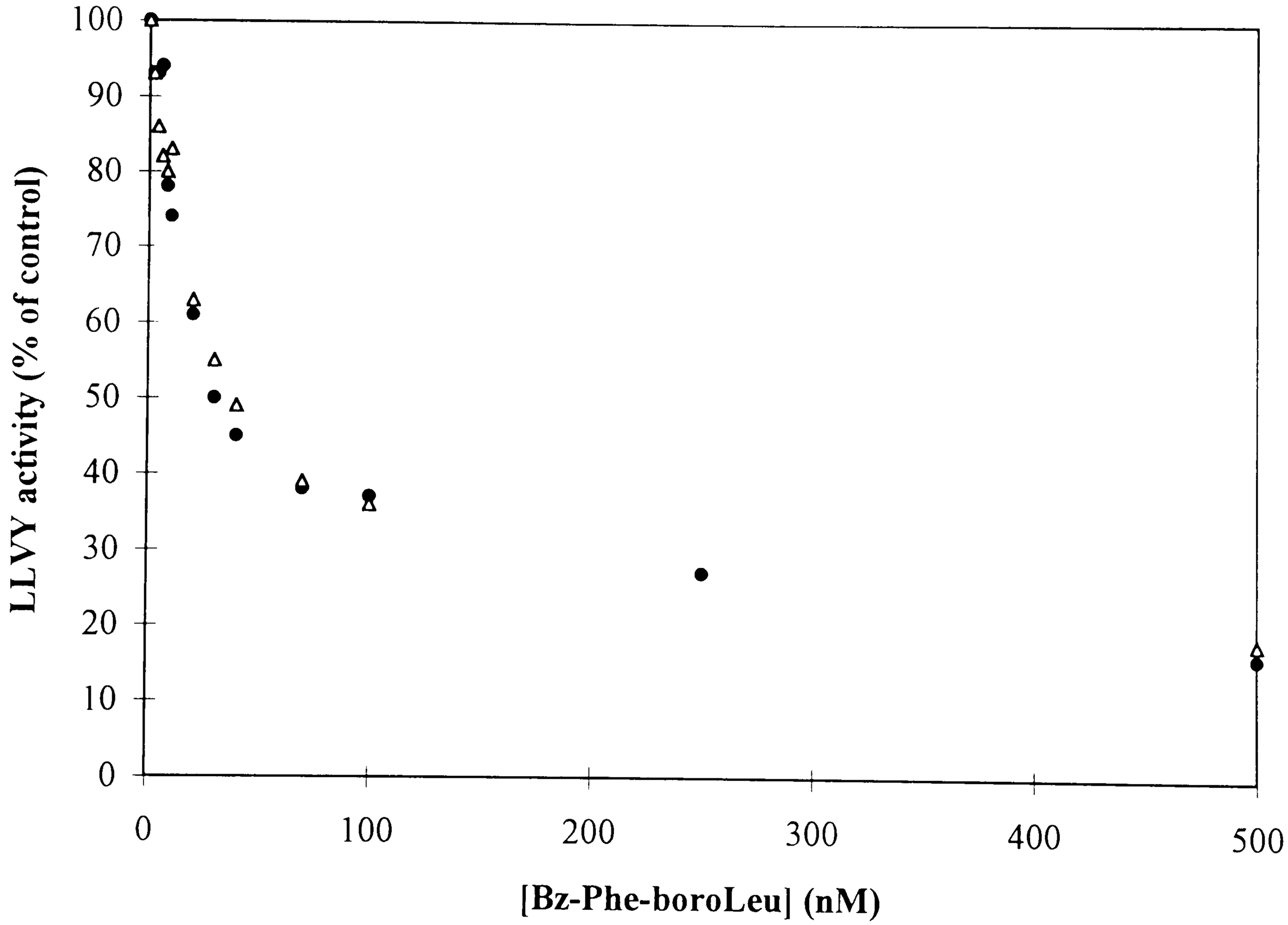
0.8
1.0
[Cbz-Leu-Leu-boroLeu] (μM)


LLVY activity (% of control)
100
90
80
70
60
50
40
30
20
10
0
0
100
200
300
400
500
[Bz-Phe-boroLeu] (nM)

The equation produced a good fit to the whole of the data sets for compound 1 and Bz-Phe-boroLeu (pinane diol ester). One of the curve fits for compound 1 is shown in Figure 3.6 (page 91). However, a curve fit could not be generated by Scientist 2.0 using certain sets of data obtained with 2 μg liver 20S proteasome instead of 1 μg. This was the case for compound 1 but not for Bz-Phe-boroLeu (pinane diol ester). Also the effect of compound 1 on the LLVY activity of liver 20S proteasomes was determined using 10 or 20 μM substrate rather than 40 μM. Figures 3.7A and 3.7B (pages 92, 93) illustrate the competitive curve fits to a set of data obtained using 10 or 20 μM Suc-LLVY-AMC. Figures 3.8A and 3.8B (pages 94, 95) illustrate the corresponding non-competitive curve fits. The Ki values generated by the competitive equation over a 0-40 nM compound 1 range, were 8 and 11 nM for 10 and 20 μM substrate respectively (Table 3.6, page 96). These values compared well with a Ki value of 19 nM determined for two experiments using 40 μM substrate. Ki values generated by the non-competitive equation for experiments using 10 and 20 μM substrate were significantly lower at 2 and 4 nM respectively (Table 3.6). Analysis of the inhibition data clearly indicated that Ki values of 2 or 4 nM were not reasonable. At these compound 1 concentrations the LLVY activity was inhibited by less than 20%. Ki values of 8 or 11 nM were feasible because the LLVY activity was inhibited by approx. 40%. Therefore the noncompetitive model was discarded at this stage.

Figure 3.6 - An equation that describes non-competitive tight-binding inhibition produces a good fit to LLVY inhibition data over a large compound 1 concentration range (page 91)

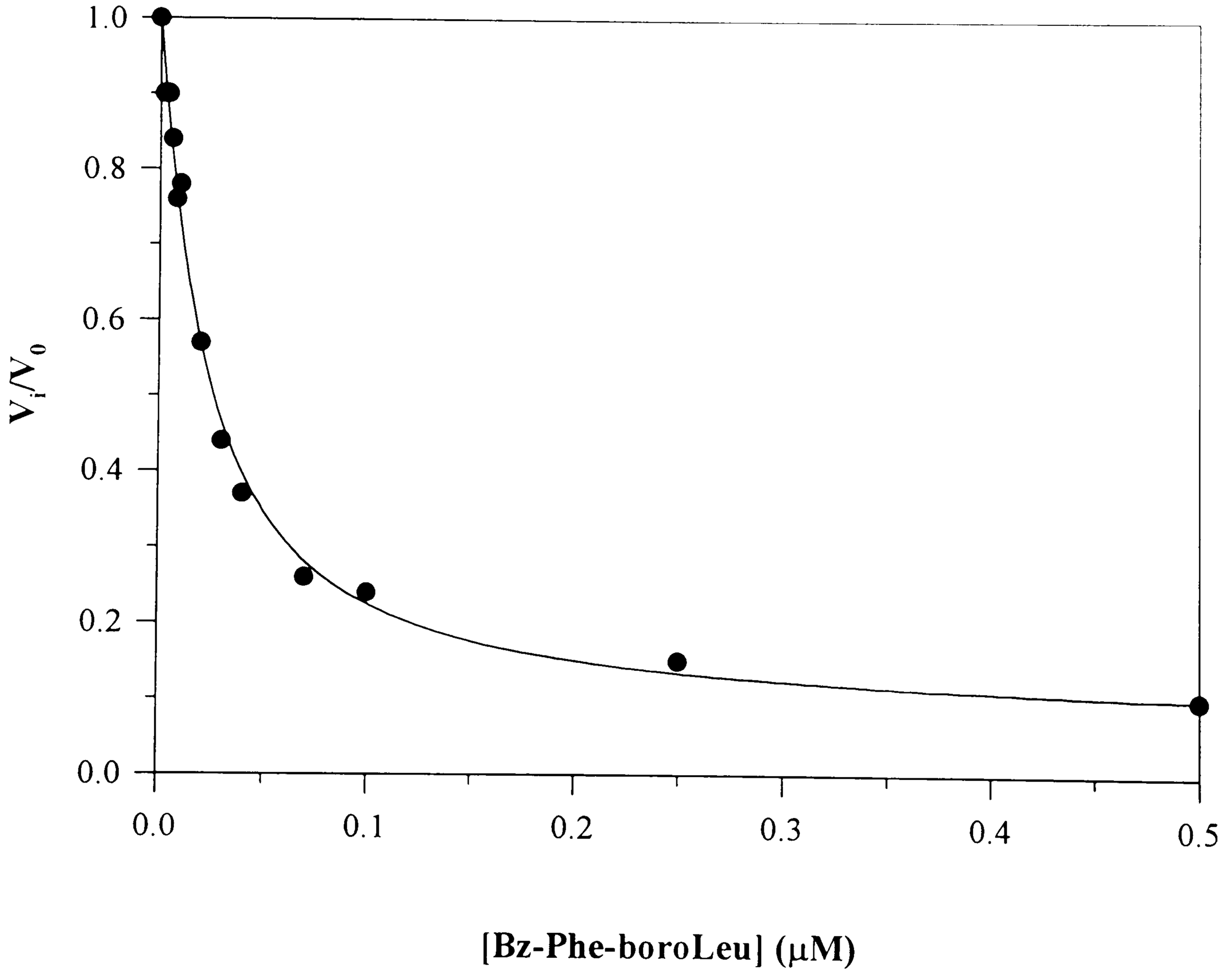
The experimental protocol is described in the legend to Figure 3.3. The non-competitive equation for tight-binding inhibitors was used to generate a curve fit to the data.

Figure 3.7 - Competitive equation curve fits to data describing the inhibition of the liver 20S proteasome LLVY activity by Bz-Phe-boroLeu (compound 1) measured with 10 or 20 μM Suc-LLVY-AMC

Experiments were performed and the data analysed as described in the legend to Figure 3.3 using 10 μM (Panel A) or 20 μM (Panel B) Suc-LLVY-AMC as substrate.
Panel A on page 92. Panel B on page 93.

Figure 3.8 - Non-competitive equation curve fits to data describing the inhibition of the liver 20S proteasome LLVY activity by Bz-Phe-boroLeu (compound 1) measured with 10 or 20 μM Suc-LLVY-AMC

Experiments were performed and the data analysed as described in the legend to Figure 3.6 using 10 μM (Panel A) or 20 μM (Panel B) Suc-LLVY-AMC as substrate.
Panel A on page 94. Panel B on page 95.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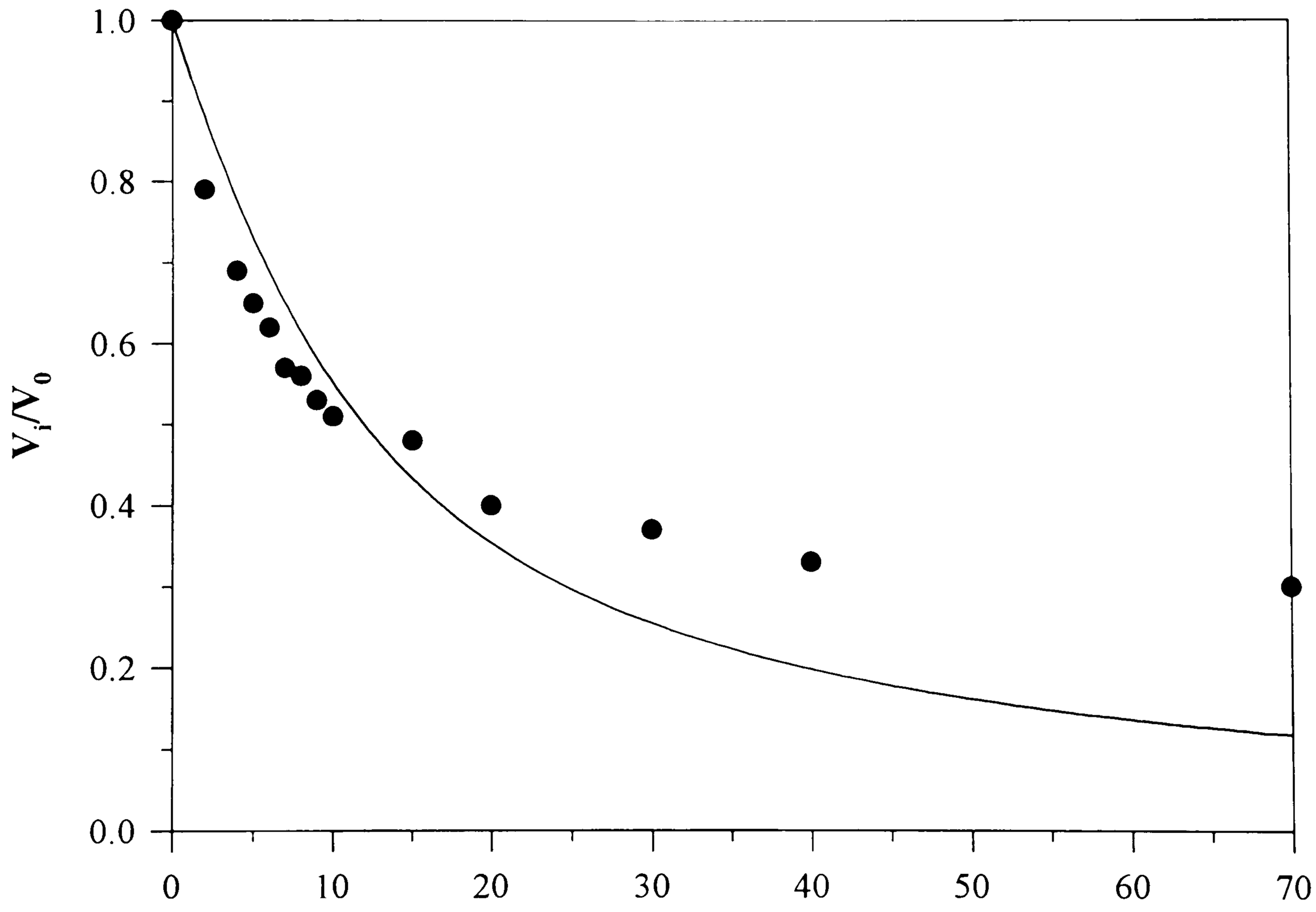
0.5
[Bz-Phe-boroLeu] (μM)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0.4
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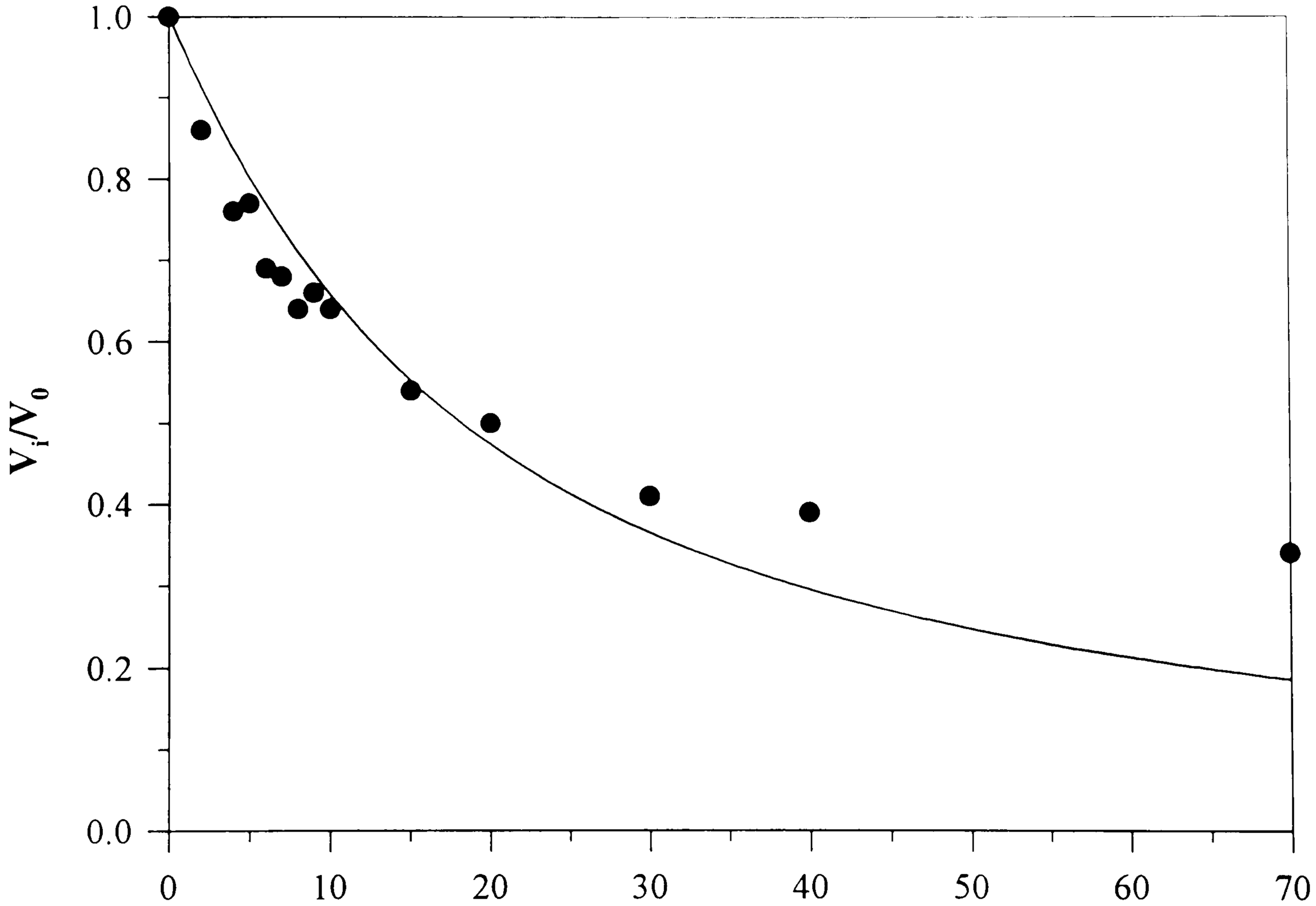
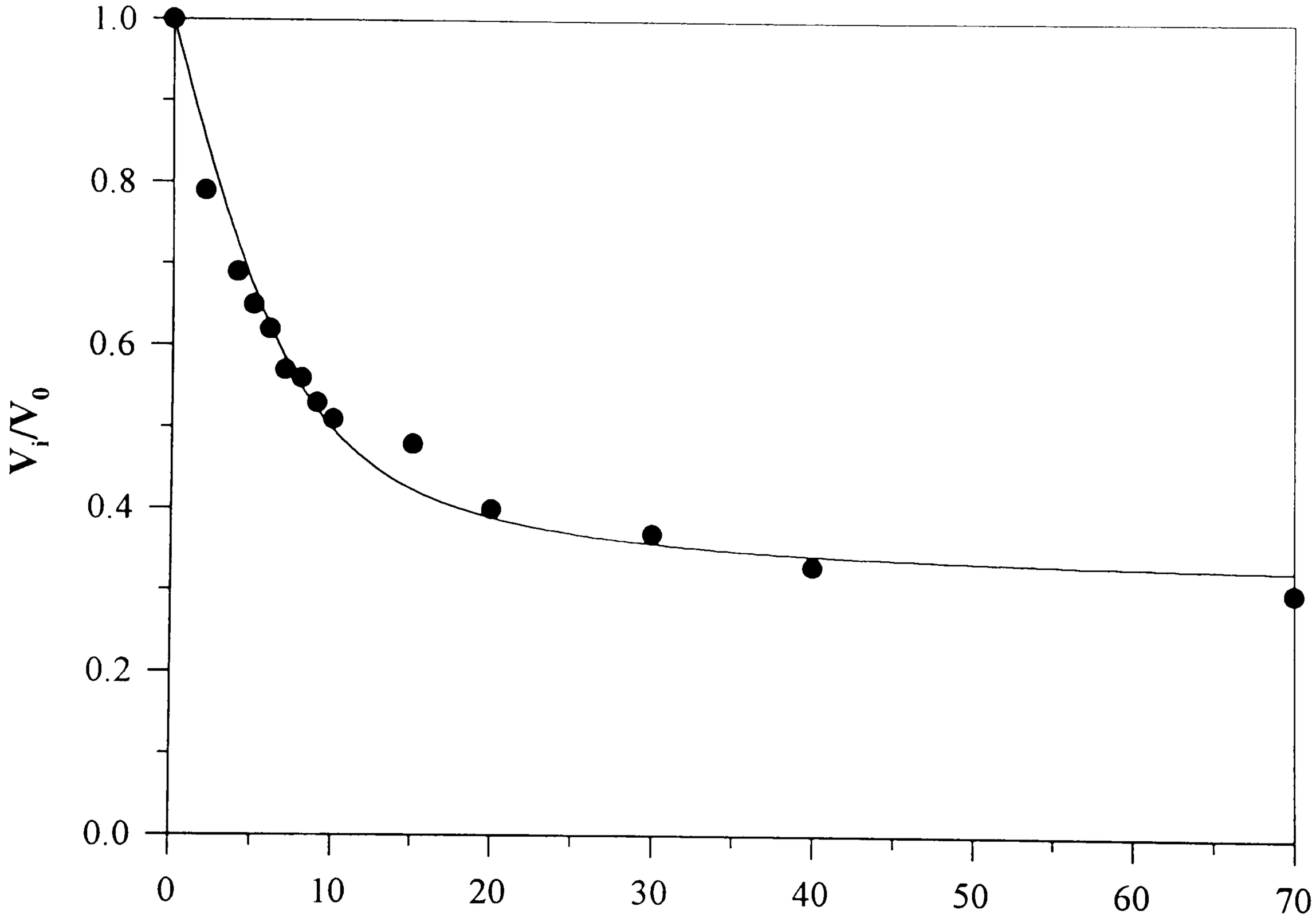
0
10
20
30
40
50
60
70
[Bz-Phe-boroLeu] (nM)


1.0
0.8
0.6
0.4
0.2
0.0
V_i/V_0
0
10
20
30
40
50
60
70
[Bz-Phe-boroLeu] (nM)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0.8
0.6
0.4
0.2
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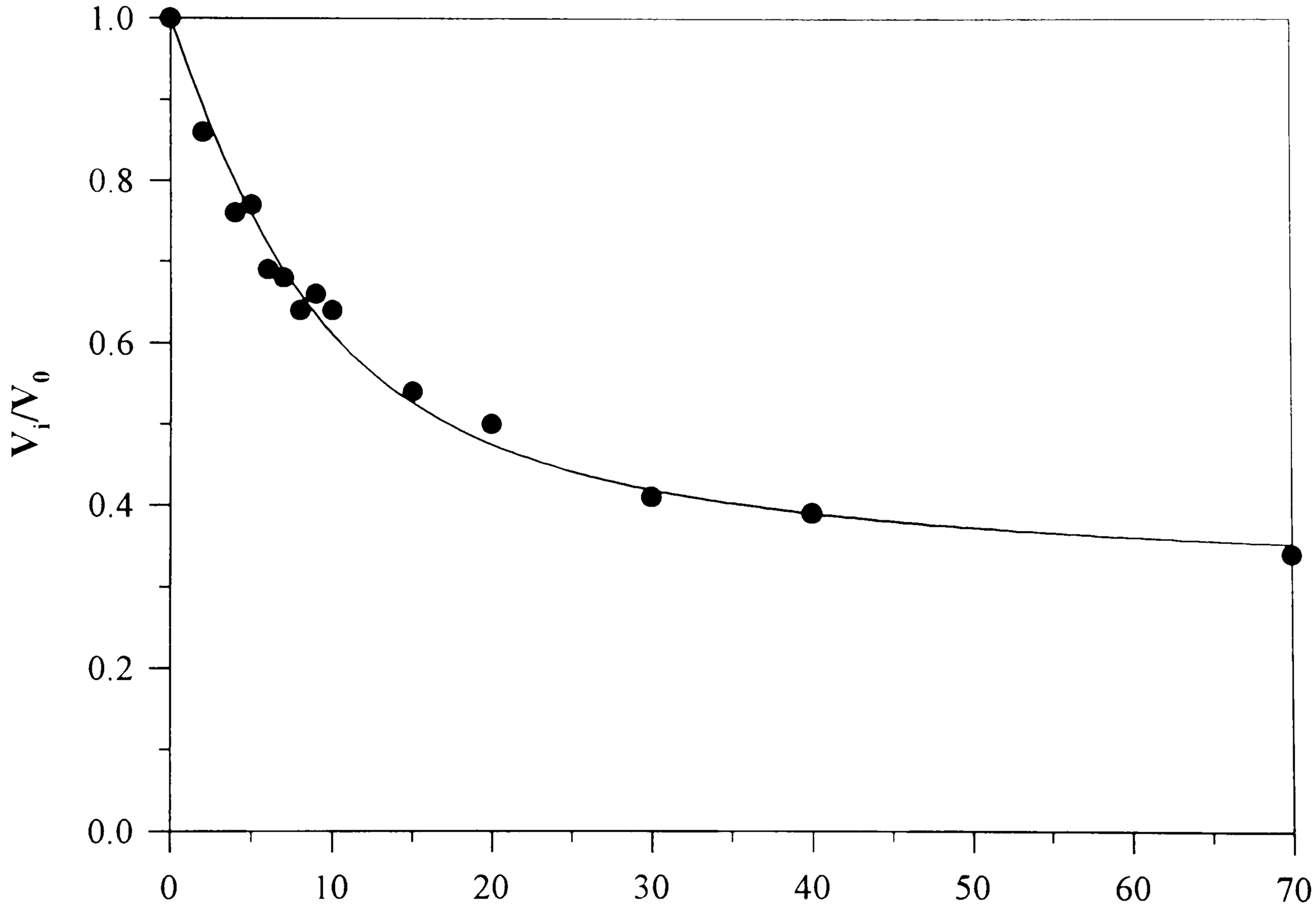
V_i/V_0
0
10
20
30
40
50
60
70
[Bz-Phe-boroLeu] (nM)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0.8
0.6
0.4
0.2
0.0
V_i/V_0
0
10
20
30
40
50
60
70
[Bz-Phe-boroLeu] (nM)

Table 3.6 - Summary of Ki values determined for compound 1

Model of inhibition	Substrate concentration (μM)	Ki value (nM)
Competitive tight-binding Henderson equation	10	8 (range ± 1)
	20	11 (range ± 1)
	40	19 (range ± 2)
Non-competitive tight-binding equation	10	2 (range ± 0)
	20	4 (range ± 1)
	40	31 (range ± 0)
Partially competitive tight-binding equation	10	4 (range ± 0)
	20	8 (range ± 1)
	40	25 (range ± 0)

Assays were performed as described in Chapter 2 using 1 μg of liver 20S proteasome. Ki values were determined by fitting data from separate experiments performed in duplicate, to the three equations describing either competitive, non-competitive or partially competitive tight-binding inhibition. Curve fits were generated using a limited 0-40 nM compound 1 concentration range. Ki values are given as the mean ± range from two separate experiments.

3.6.1 - Inhibition data were fitted to a partially competitive model

Discussions with Prof. Rob John lead to a proposal that the observed inhibition could be partially competitive inhibition. In this scenario, substrate can bind to and be hydrolysed by 20S proteasome and 20S proteasome-inhibitor complexes. An equation that represents this type of inhibition was written by Prof. Rob John and is shown in Chapter 2. The substrate was proposed to bind less efficiently to the 20S proteasome-inhibitor complex than the 20S proteasome. Hence Km and Kmi values were used in the equation. Ki values for peptidyl boronic acid binding to the 20S proteasome were very much lower than the Km for the LLVY activity. Therefore only one Ki value was used in the equation. This type of inhibition was thus termed tight-binding partially competitive inhibition. This model was attractive because it could explain why the inhibition failed to extend past approximately 90%, even at 30 × Ki. Initial fits to sets of data for compound 1 using 1 μg liver 20S proteasome and 40 μM substrate were good, over a large range of inhibitor concentrations. One of these fits is shown in Figure 3.9A (page 100). Figures 3.9B and 3.9C (pages 101, 102) illustrate curve fits using the partially competitive equation and sets of data obtained using 1 μg liver 20S proteasome and either 10 or 20 μM substrate. The Ki values using 10, 20 and 40 μM substrate over a limited (0-40 nM) inhibitor range were 4, 8 and 25 nM respectively (Table 3.6). The value of 4 nM was unacceptably low with only 20-25% inhibition being recorded at this concentration. Moreover, the calculated value of Kmi varied from 7 μM for experiments with 10 μM substrate up to several hundred or even thousands of μmol/l from experiments with 40 μM substrate. This constant also changed significantly with the value inserted into the parameter file. Rough estimates of Ki, Kmi and r were inserted into the parameter file to assist the curve fitting process. Estimates of these constants were then generated by the program. In addition,

the term r that is the difference (expressed as a fraction) in catalytic activity between the EIS and ES complexes was often estimated to be negative rather than positive. Therefore this partially competitive model was judged to be unsatisfactory.

3.6.2 - Inhibition data were fitted to a subpopulation model

One further model was considered. This assumed that a subpopulation of 20S proteasomes was present whose LLVY activity could not be inhibited by the peptidyl boronic acids. This could occur for example if a subpopulation of 20S proteasomes had an unusual arrangement of catalytic subunits. Inhibition data for compound 1 and Bz-Phe-boroLeu (pinane diol ester) were processed with the equation given in Chapter 2. This involved subtracting the fraction of activity (V_i/V_0) remaining using the highest inhibitor concentration from each initial V_i/V_0 result. These results were then expressed as a fraction of the LLVY activity that could be inhibited. The competitive Henderson equation was then used to generate a curve fit. Initial fits for experiments using 1 μg liver 20S proteasome and 40 μM substrate were good over a large range of inhibitor concentrations. However, the curve fits obtained for experiments using lower [Suc-LLVY-AMC] or 2 μg 20 proteasome were unconvincing. One of these curve fits from an experiment using 2 μg liver 20S proteasome and 0-500 nM compound 1 is shown in Figure 3.10 (page 103).

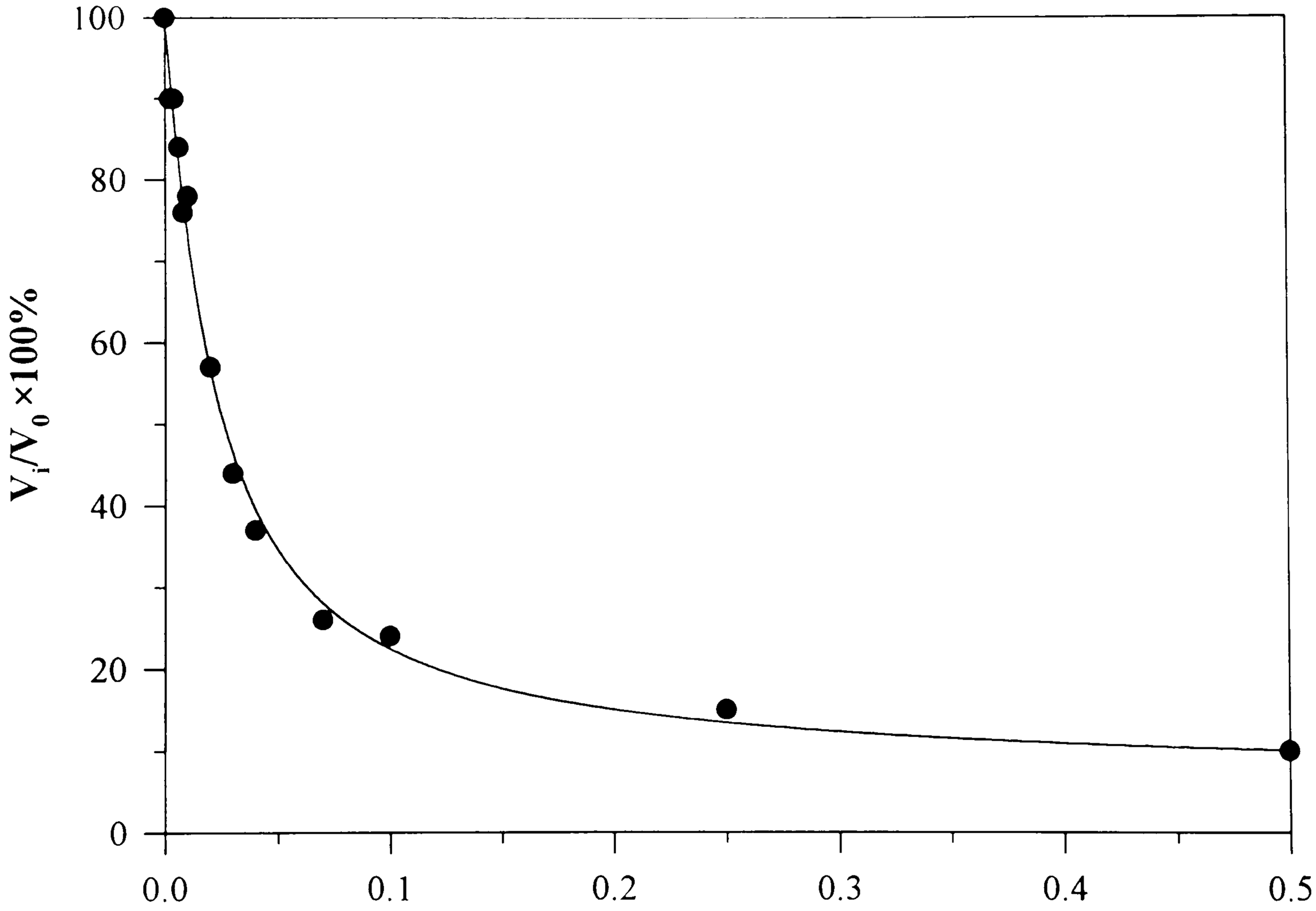
Figure 3.9 - A partially competitive tight-binding equation can generate curve fits to data describing the inhibition of the liver 20S proteasome LLVY activity by Bz-Phe-boroLeu (compound 1)

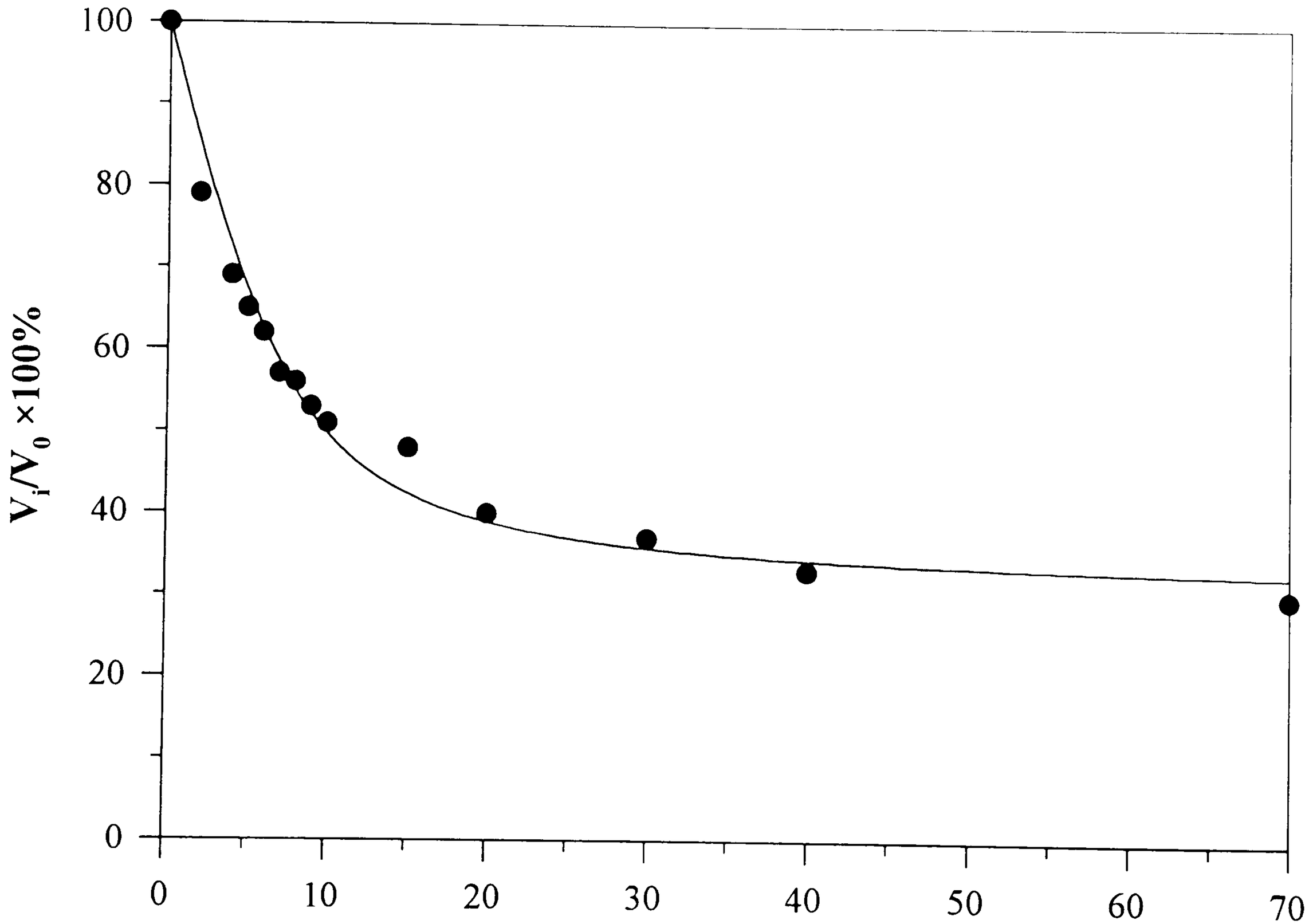
The experimental protocol is described in the legend to Figure 3.3. The V_i/V_0 values were expressed as percent rather than fraction of control before data analysis. The partially competitive equation was used to generate a curve fit to data from experiments using 40 μM (Panel A), 10 μM (Panel B) or 20 μM (Panel C) Suc-LLVY-AMC as substrate.

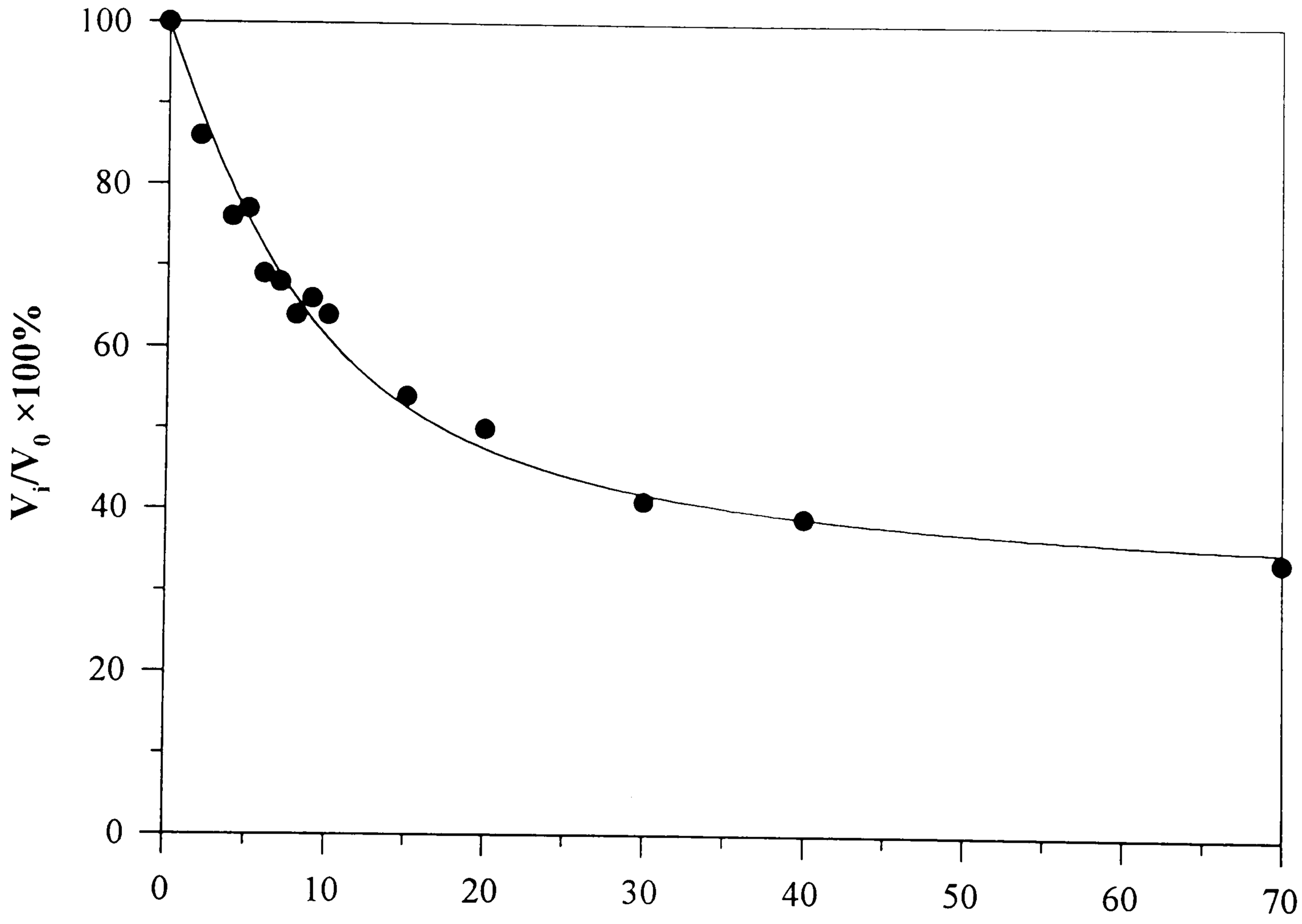
Panel A on page 100. Panel B on page 101. Panel C on page 102.

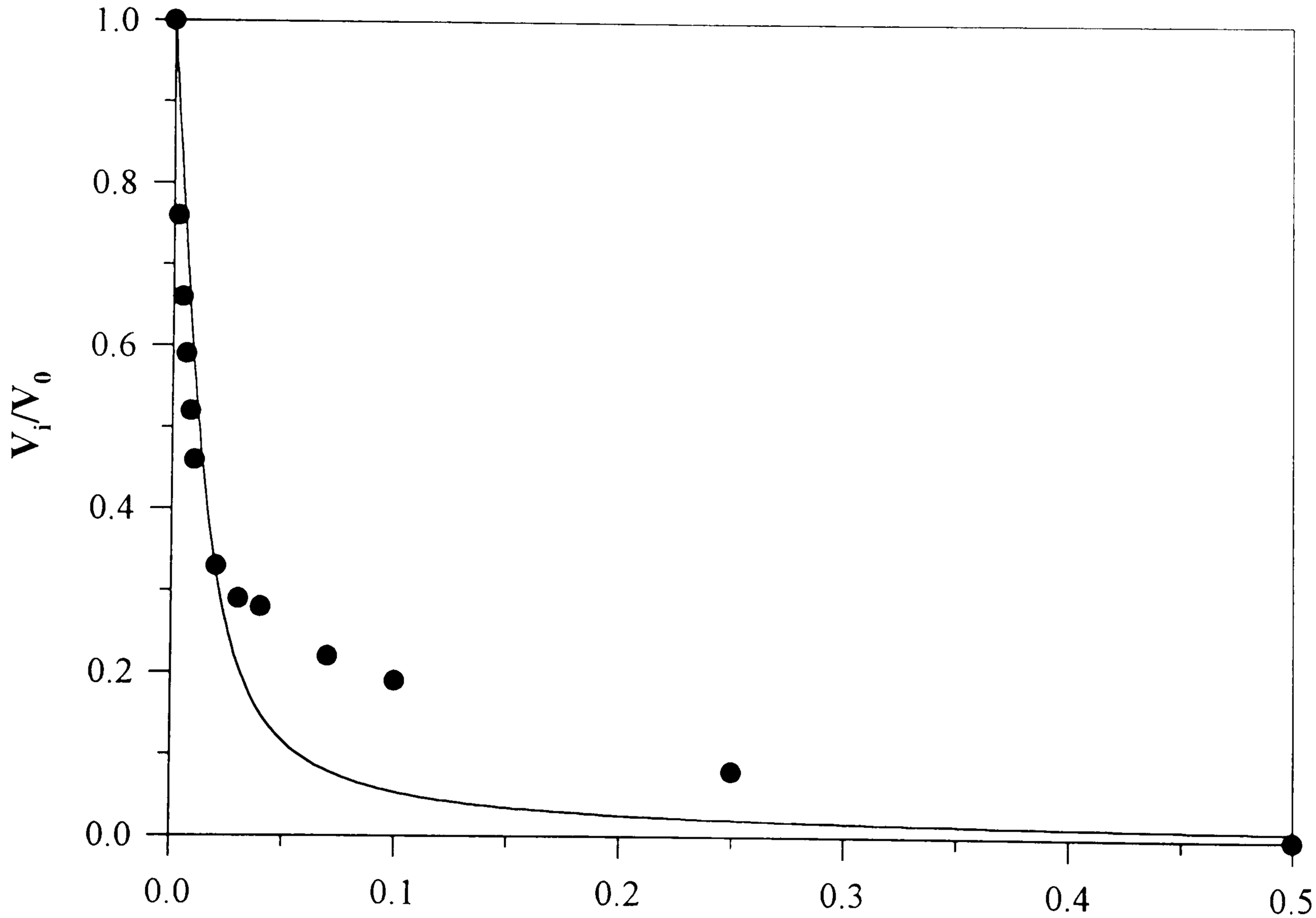
Figure 3.10 - Subpopulation model curve fit to data describing the inhibition of the liver 20S proteasome LLVY activity by Bz-Phe-boroLeu (compound 1) (page 103)

Liver 20S proteasome (2 μg) was incubated in the presence of a range of compound 1 concentrations in 50 mM Hepes buffer/KOH, pH 7.5, for 15 minutes at 25°C. Substrate (40 μM Suc-LLVY-AMC) was added and the assays were incubated at 37°C for 15 minutes. Values are from a typical experiment performed in duplicate. The initial V_i/V_0 values were first processed using the equation for the subpopulation model given in Chapter 2. The competitive Henderson equation for tight-binding inhibitors was then used to generate a curve fit through the calculated activities.


100
80
60
40
20
0
0.0
0.1
0.2
0.3
0.4
0.5
$V_i/V_0 \times 100\%$
[Bz-Phe-boroLeu] (μM)


100
80
60
40
20
0
$V_i/V_0 \times 100\%$
0
10
20
30
40
50
60
70
[Bz-Phe-boroLeu] (nM)


100
80
60
40
20
0
0
10
20
30
40
50
60
70
$V_i/V_0 \times 100\%$
[Bz-Phe-boroLeu] (nM)


1.0
0.8
0.6
0.4
0.2
0.0
V_i/V_0
0.0
0.1
0.2
0.3
0.4
0.5
[Bz-Phe-boroLeu] (μM)

3.6.3 - Further experimental work strengthens the case for a competitive model of inhibition and rules out non-competitive and partially competitive models

At this stage the competitive Henderson equation was producing the best fits to data from all four assay conditions, using a limited inhibitor concentration range. Figure 3.11 shows a competitive Henderson equation fit to the data used in Figure 3.3, over a limited 0-40 nM compound 1 concentration range. Panel A of Table 3.7 shows the results of an experiment designed to determine the exact mechanism of inhibition. At 130 and 300 μM Suc-LLVY-AMC concentrations the level of inhibition was decreased across the range of compound 1 concentrations. This rules out a non-competitive mechanism that would be unaffected by increases in substrate concentration. Ki values were calculated at the three different substrate concentrations using the competitive Henderson equation. These Ki values are shown in panel B of Table 3.7. Over the full compound 1 concentration range the Ki values were between 16 and 18 nM. However, the curve fits were below the data points at high compound 1 concentrations. The Ki values estimated over a limited 0-2×Ki concentration range were also in good agreement at 13-16 nM.

The effect of 1-10 μM compound 1 on the LLVY activity of 1 μg liver 20S proteasome was subsequently determined. A substrate concentration of 40 μM was used in the assays. In both determinations the LLVY activity was inhibited 95% and 97% using 1 and 2 μM compound 1 respectively. 10 μM compound 1 caused 98% inhibition of the LLVY activity. In the partially competitive model, 20S proteasomes with bound inhibitor could still hydrolyse substrate to a limited extent. Therefore these results rule out the partially competitive mechanism. In addition the subpopulation model listed above can be rejected. The LLVY

activity of the 20S proteasome subpopulation should continue to be resistant to inhibition, as the compound 1 concentration increases to 10 μM. This was not shown to be the case.

Figure 3.11 - The competitive Henderson equation produces a good fit to LLVY inhibition data over a limited Bz-Phe-boroLeu (compound 1) concentration range (page 106)

The experimental protocol is described in the legend to Figure 3.3. The competitive Henderson equation was used to generate a curve fit over a limited (0-40 nM) inhibitor concentration range.

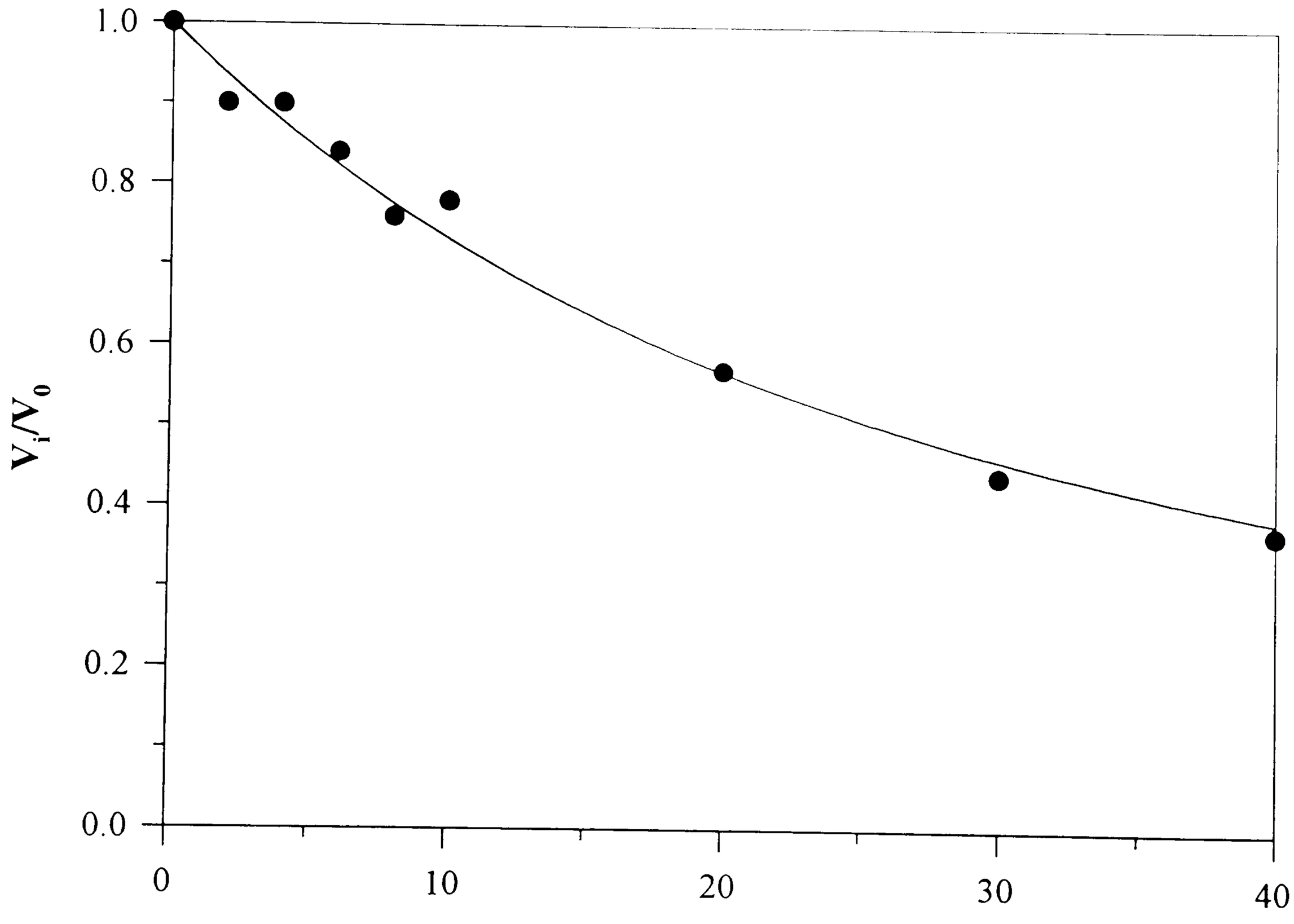

1.0
0.8
0.6
0.4
0.2
0.0
V_i/V_0
0
10
20
30
40
[Bz-Phe-boroLeu] (nM)

Table 3.7 - Inhibition of liver 20S proteasomes by compound 1 at different concentrations of the substrate, Suc-LLVY-AMC

Panel A

[Suc-LLVY-AMC] (μM)	LLVY activity (% of control) at [compound 1] (nM)							
	0	8	14	20	40	80	150	300
40	100	80	65	50	35	29	20	14
130	100	81	73	62	49	32	29	22
300	100	71	75	76	57	42	33	25

Assays were performed as described in Chapter 2 using 1 μg of liver 20S proteasome. The values for 150 nM compound 1 were obtained from a single experiment performed in duplicate. The other values are given as the average of two experiments performed in duplicate. These values are expressed as percent of control activity in samples containing no inhibitor. The six sets of data were individually fitted to the Henderson equation for competitive tight-binding inhibitors. The Ki values for the full 0-300 nM and shortened 0-40 nM range of concentrations are shown in panel B. These values are given as the mean ± the range for the two separate experiments.

Panel B

[Suc-LLVY-AMC] (μM)	Ki (nM)	
	0-300 nM compound 1	0-40 nM compound 1
40	17 ± 4	15 ± 4
130	18 ± 2	16 ± 5
300	16 ± 1	13 ± 2

The best model to describe the observed inhibition of the LLVY activity was therefore chosen to be the competitive tight-binding model. NMR spectroscopy was previously used to show that substrate analogue peptidyl boronic acids covalently modify the catalytic serine of α-lytic protease (Bachovchin et al., 1988). The use of the model was limited to peptidyl boronic acid concentrations of 0 to 2×Ki. The deviation from ideal behaviour above 2×Ki was presumably due to the multicatalytic nature of the 20S proteasome. Each 20S proteasome contains at least two sites that are proposed to cleave the Suc-LLVY-AMC substrate. It is not known whether both of these catalytic sites are fully operational at any one time. It maybe the case that binding of inhibitor to one of these sites obstructs the binding of inhibitor to the other site. This phenomenum was observed with AAF-chloromethyl ketone where only 50% of the LLVY activity could be inhibited (Reidlinger et al., 1997). Hydrolysis of Suc-LLVY-AMC will occur to some extent at all the 20S proteasome catalytic sites. Modification of the main LLVY hydrolysing sites by peptidyl boronic acid, may cause conformational changes that increase the extent of LLVY hydrolysis at other sites. This would mean the apparent inhibition was less than the extent of peptidyl boronic acid modification. Peptidyl boronic acids will also modify the main trypsin-like and PGPH catalytic sites. Modification of these sites will tend to be greater at inhibitor concentrations above the Ki for the LLVY activity (see Tables 3.2A and 3.2B). Resultant conformational changes may inhibit or activate hydrolysis at the main LLVY hydrolysing sites, further complicating the inhibition data.

3.7 - Compound 1 causes potent inhibition of activated 20S proteasomes

Early studies on 20S proteasomes showed that 0.01-0.02% SDS stimulated the chymotrypsin-like and PGPH activities. The trypsin-like activity was either stimulated or inhibited depending on the substrate and assay conditions used (Rivett et al., 1989; Arribas & Castaño, 1990).

Higher concentrations of SDS e.g. 0.1% caused complete inhibition of the peptidase activities. SDS was proposed to stimulate or inhibit peptidase activities by causing conformational changes. The effects of SDS could be reversed by simple dilution suggesting that the 20S proteasome was not dissociating at higher SDS concentrations (Arribas & Castaño, 1990). Some research groups have chosen to investigate the effect of enzyme inhibitors on the 20S proteasome in assay buffers containing low concentrations of SDS. For example Ki values for the effect of peptidyl aldehydes on the 20S proteasome LLVY and LLE activities were determined in an assay buffer containing 0.035% SDS (Rock et al., 1994). Also K_{obs}/[I] values for the effect of peptidyl vinyl sulphones on 20S proteasome activities, were determined in a Hepes assay buffer containing 0.01% SDS (Bogyo et al., 1998). Figure 3.12 shows the effect of compound 1 on the LLVY activity of 1 μg liver 20S proteasome in the absence or presence of 0.02% SDS. Initial examination of the data suggested that the Ki value in the presence of SDS was 25-30 nM. Therefore the Ki value was not significantly altered by the presence of 0.02% SDS. What was interesting was the rapid decrease in percent activity values between 20 and 100 nM compound 1. The inhibition was essentially complete using 100 nM compound 1. This showed that 0.02% SDS was assisting the binding of compound 1 to one or both of the proposed chymotrypsin-like catalytic sites. Binding of one molecule compound 1 to one of the catalytic sites may inhibit the binding of another molecule of compound 1 to the second site. Greater conformational flexibility induced by the presence of 0.02% SDS may assist the modification of the second site.

The level of AMC produced by the 20S proteasome in the presence of 0.02% SDS was eight times higher than normal. It was proposed that product inhibition may be assisting the inhibition of the LLVY activity by compound 1. Therefore the assay conditions were changed by using 0.5 μg aliquots of liver 20S proteasome and an assay period of 6 minutes. These

changes were designed to reduce the AMC production to 1.6 times the normal level in assay buffer containing 0.02% SDS. The results of this experiment are shown in Figure 3.13. The AMC production was reduced to the predicted level in the presence of 0.02% SDS. Compound 1 was shown to cause potent inhibition of the LLVY activity both in the absence and presence of 0.02% SDS. In the absence of SDS the Ki was slightly higher than anticipated at 53 nM (Figure 3.14). In the presence of SDS the Ki was estimated to be 10 nM with inhibition being virtually complete at 50 nM compound 1.

Figure 3.12 – SDS (0.02%) increases the extent of inhibition of the 20S proteasome LLVY activity above Ki concentrations of compound 1 (page 112)

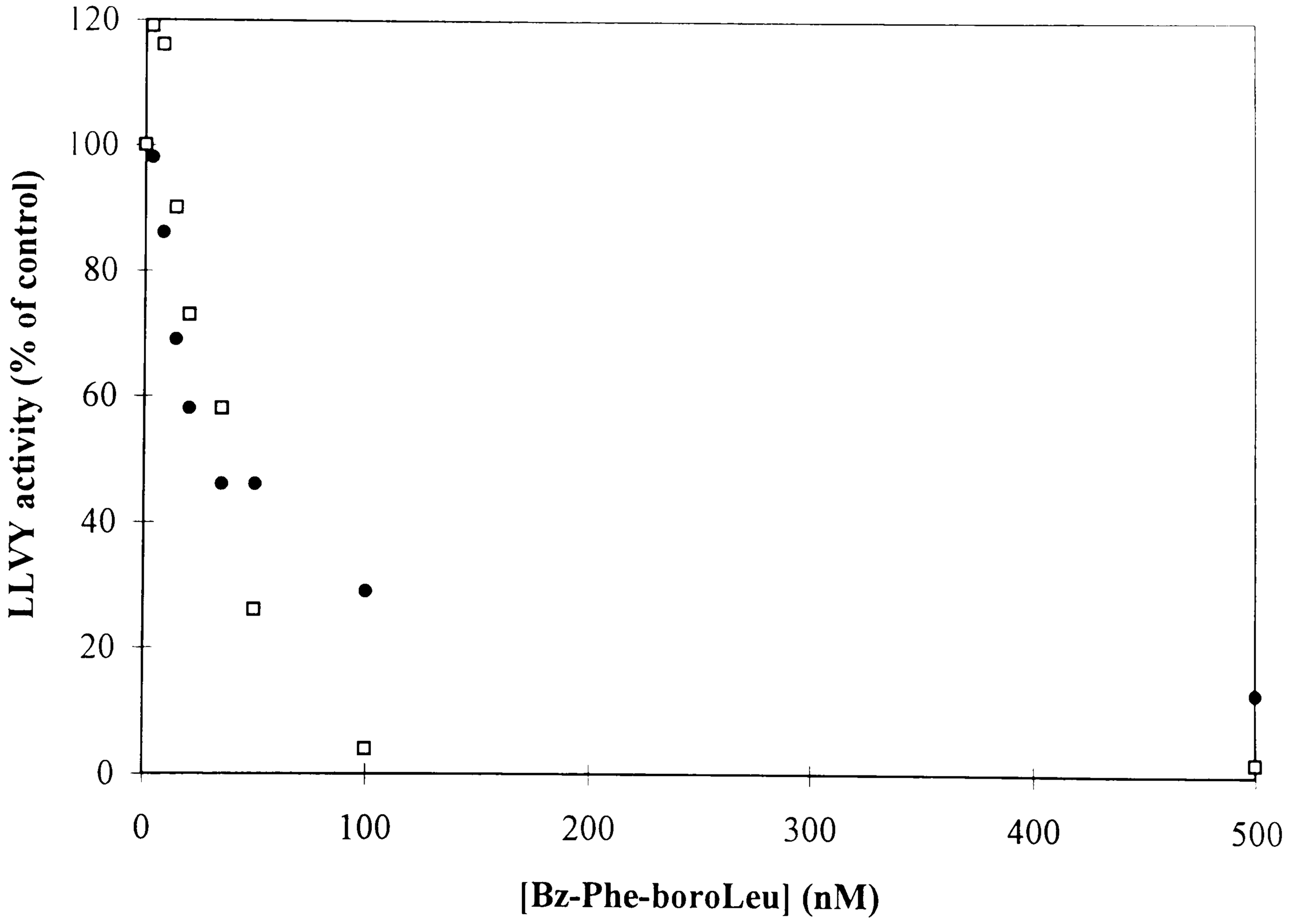
Liver 20S proteasome (1 μg) was incubated with selected concentrations of compound 1 in 50 mM Hepes buffer/KOH, pH 7.5 containing no (filled circles) or 0.02% (open squares) SDS. This preincubation was conducted for 15 minutes at 25°C. Substrate (40 μM Suc-LLVY-AMC) was added and the assays were incubated at 37°C for 15 minutes, as described in Chapter 2. The values are from a single experiment conducted in duplicate. These values are expressed as percent of control activity in samples containing no inhibitor.

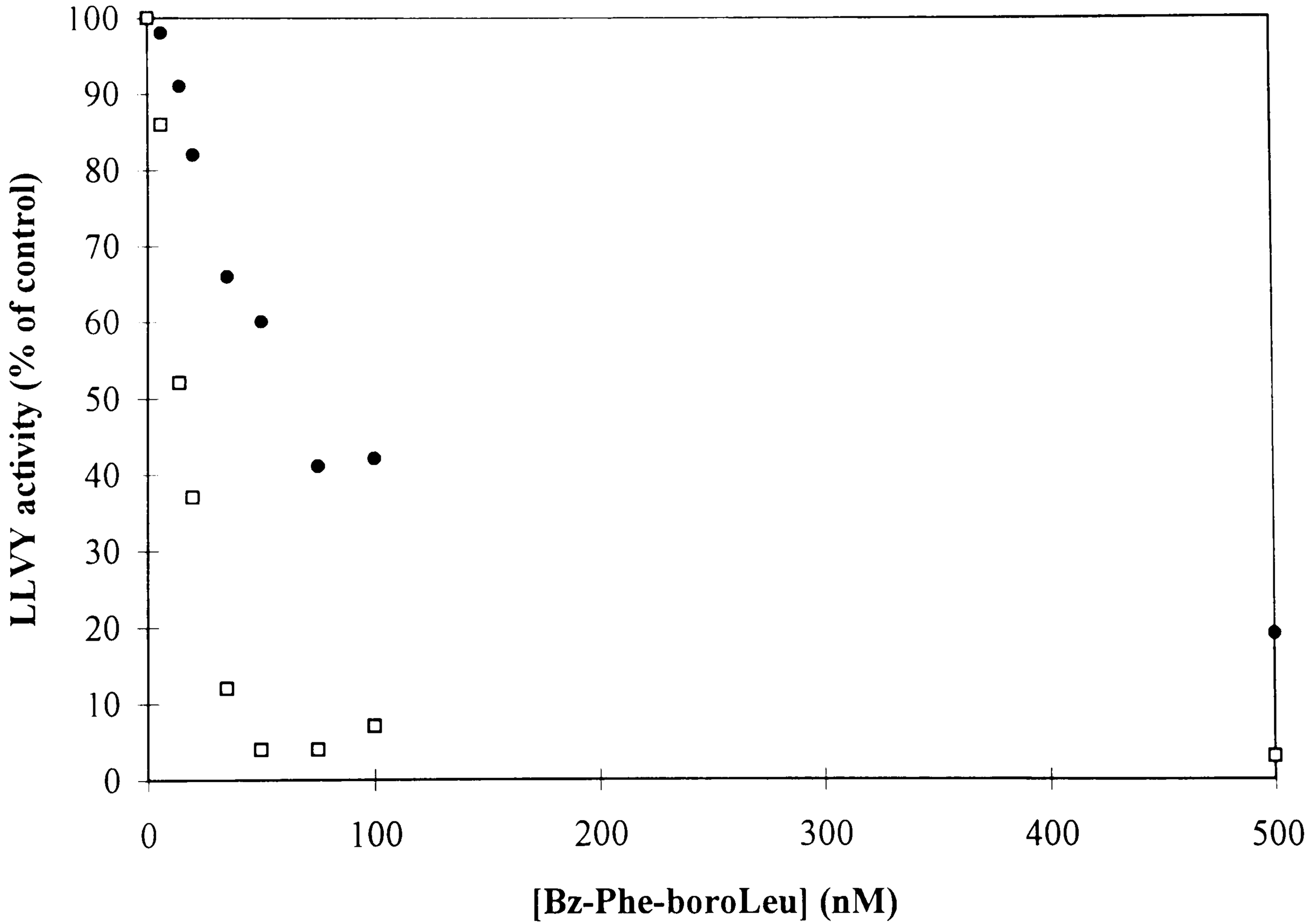
Figure 3.13 – SDS (0.02%) increases the extent of inhibition of the 20S proteasome LLVY activity above Ki concentrations of compound 1 using half the amount of 20S proteasome and a shorter assay time (page 113)

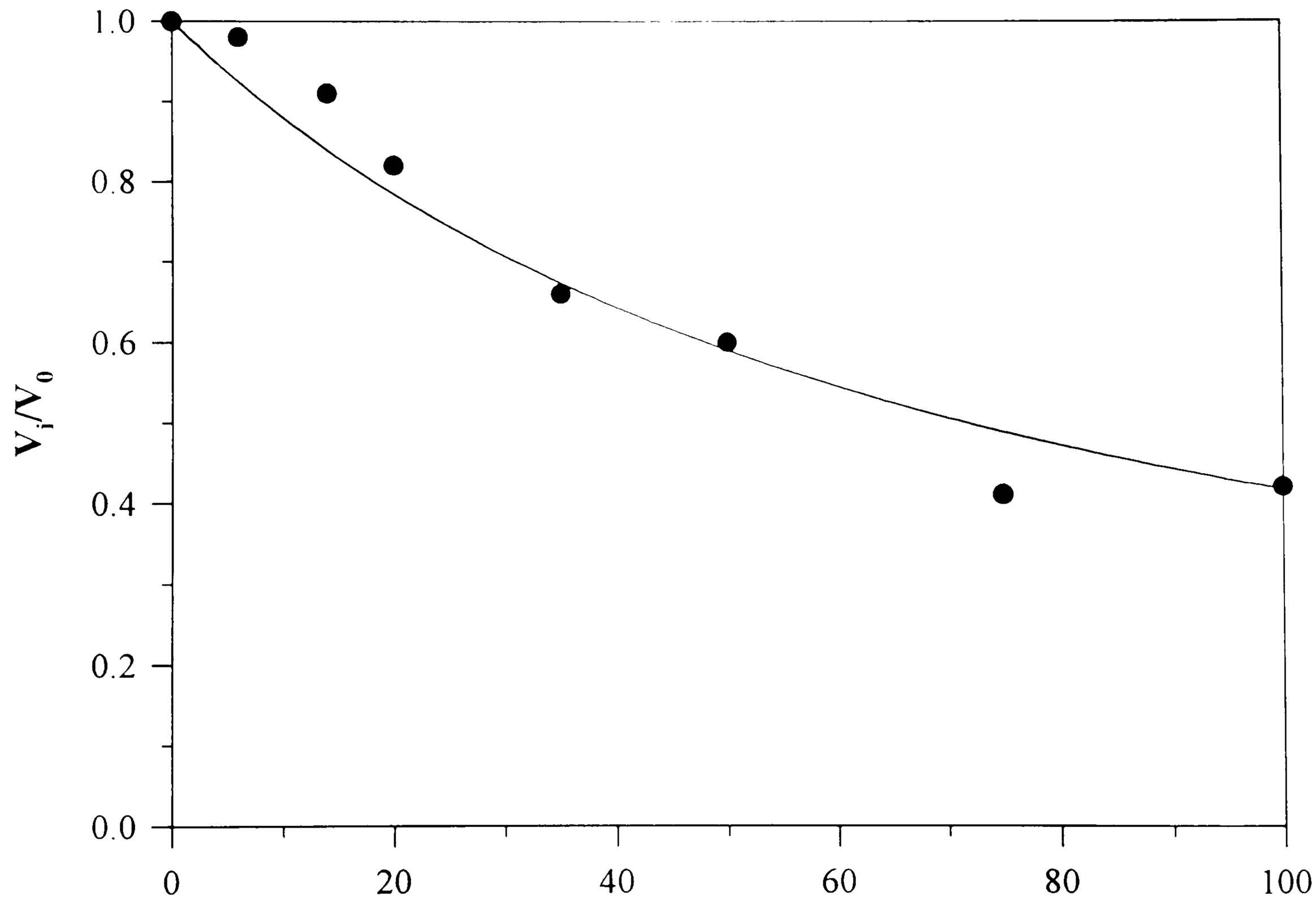
Liver 20S proteasome (0.5 μg) was incubated with selected concentrations of compound 1 in 50 mM Hepes buffer/KOH, pH 7.5 containing no (filled circles) or 0.02% (open squares) SDS. This preincubation was conducted for 15 minutes at 25°C. Substrate (40 μM Suc-LLVY-AMC) was added and the assays were incubated at 37°C for 6 minutes. Values are from a single experiment conducted in duplicate. These values are expressed as percent of control activity in samples containing no inhibitor.

Figure 3.14 - Bz-Phe-boroLeu is a tight-binding inhibitor of the 20S proteasome LLVY activity at low (3.5 nM) proteasome concentrations (page 114)

The experimental protocol is described in the legend to Figure 3.13. The data obtained in the absence of SDS were fitted to the competitive Henderson equation over a limited 0-100 nM compound 1 concentration range.


LLVY activity (% of control)
120
100
80
60
40
20
0
0
100
200
300
400
500
[Bz-Phe-boroLeu] (nM)


LLVY activity (% of control)
100
90
80
70
60
50
40
30
20
10
0
0
100
200
300
400
500
[Bz-Phe-boroLeu] (nM)


1.0
0.8
0.6
0.4
0.2
0.0
V_i/V_0
0
20
40
60
80
100
[Bz-Phe-boroLeu] (nM)

3.8 - Compound 1 is a potent inhibitor of the 26S proteasome LLVY activity

Dr Steve Assinder characterised the effects of a number of peptidyl boronic acids on the 26S proteasome. He showed that peptidyl boronic acids were slow binding reversible inhibitors of the 26S proteasome LLVY activity. Experiments were performed to determine the effect of 0-200 nM compound 1 on the liver 26S proteasome LLVY activity. 26S proteasome (0.1 μg; 0.25 nM) and 40 μM Suc-LLVY-AMC were used in these assays. The Henderson equation for competitive tight-binding inhibition is applicable to situations where the total enzyme concentration divided by the Ki is between 0.01 and 100 (Henderson, 1972). When the ratio is below 0.01, virtually all the inhibitor molecules are free and the Michaelis-Menten equation for competitive inhibitors is then applicable (Henderson, 1972). The competitive Henderson equation gave a good fit to the data over the full 0-200 nM concentration range. The Ki was determined to be 11 ± 6 nM from three separate determinations. The E_{total}/Ki ratio was 0.02, which means that this equation can be correctly used with the Bz-Phe-boroLeu data.

The Ki value for inhibition of the liver 20S proteasome LLVY activity by compound 1 was determined to be 17 ± 4 nM for three separate determinations (Table 4.1). Ki values for inhibition of the 20S and 26S proteasome LLVY activities, were predicted to be similar because the same catalytic sites are present in both complexes. More surprising was the good fit of the competitive equation to experimental data that rapidly approached zero. The 26S proteasome contains two 19S regulatory complexes at the ends of the 20S proteasome. These regulatory complexes are known to assist the process of protein degradation. They may also constrain the structure of the 20S proteasome. This may mean that the catalytic sites operate in a more independent manner, being less effected by conformational changes at other sites.

3.9 - Summary

Some peptidyl boronic acids are tight-binding inhibitors of the liver 20S proteasome LLVY activity. Bz-Phe-boroLeu (compound 1), the most effective of these compounds exerts inhibition through an apparent slow binding mechanism. Bz-Phe-boroLeu (80 nM) caused significant inhibition of the LLE1 activity and stimulation of the LSTR activity. The inhibition of 20S proteasome activities was reversible during dialysis, although long time periods were often required to recover significant amounts of activity e.g. LLVY activity. Kinetic analysis showed that peptidyl boronic acid inhibition of the 20S proteasome LLVY activity, did not follow ideal behaviour. Even at high nanomolar concentrations of the tight-binding peptidyl boronic acids, it was not possible to achieve complete inhibition of the liver 20S proteasome LLVY activity. The Henderson model for competitive tight-binding inhibitors gave a good fit to the experimental data, over a limited inhibitor concentration range. Similar Ki values were determined for Bz-Phe-boroLeu inhibition of the LLVY activity of liver 20S proteasomes, liver 20S proteasomes activated with 0.02% SDS and liver 26S proteasomes.

Chapter 4 - Determining the structure-activity relationships that govern the interaction of peptidyl boronic acids with the chymotrypsin-like catalytic sites of liver and spleen 20S proteasomes

Chapter 4 - Determining the structure-activity relationships that govern the interaction of peptidyl boronic acids with the chymotrypsin-like catalytic sites of liver and spleen 20S proteasomes

4.1 - Introduction

In this chapter I will describe the determination of Ki values for inhibition of the liver 20S proteasome LLVY activity by a number of peptidyl boronic acids. All of the peptidyl boronic acids supplied by the chemists at SmithKline Beecham contained a hydrophobic P1 residue. These compounds were predicted to be most effective against the chymotrypsin-like activity. However the nature of the residues other than P1 have been shown to play important roles in directing peptide substrates and peptidyl inhibitors to particular catalytic sites (Orlowski et al., 1993; Cardozo et al., 1994; Reidlinger et al., 1997; Bogyo et al., 1998). Competition experiments using two or more peptide substrates, do not readily give information on which amino acids combinations bind effectively to catalytic sites. The peptide substrates usually exhibit a weak affinity for the catalytic sites with Km values very much greater than the enzyme concentration. Comparison of Ki values for these potent inhibitors reveals amino acid arrangements that bind particularly effectively to the chymotrypsin-like catalytic sites. Other amino acid combinations are less effective due to unfavourable non-covalent interactions with nearby protein residues. Hence these studies allow a greater understanding of the catalytic site structure particularly in conjunction with crystallographic data (Löwe et al., 1995; Groll et al., 1997). It was also of interest to determine whether pinacol ester or pinane diol ester blocked peptidyl boronic acids, were equally effective compared to the corresponding free acid. At the time these experiments were performed, cell culture studies had shown no significant difference in potency between the blocked and unblocked forms in experiments where cells were

incubated with peptidyl boronic acid for 24 hours (Christie et al., 1999). In related studies the effect of several peptidyl boronic acids on the liver 20S proteasome trypsin-like activity was determined. None of the peptidyl boronic acids were shown to be tight-binding inhibitors of this activity. The most effective of these inhibitors exhibited an IC50 value of approximately 1 μM.

In the second part of the chapter I will compare and contrast the effect of the peptidyl boronic acids on liver and spleen 20S proteasomes. Liver 20S proteasomes are constructed of approximately equal amounts of the constitutively expressed and IFN-γ inducible β catalytic subunits. Spleen 20S proteasomes in contrast are predominantly assembled with IFN-γ inducible β catalytic subunits. Hence these comparisons will illustrate how readily the two types of catalytic subunits are modified by the peptidyl boronic acids.

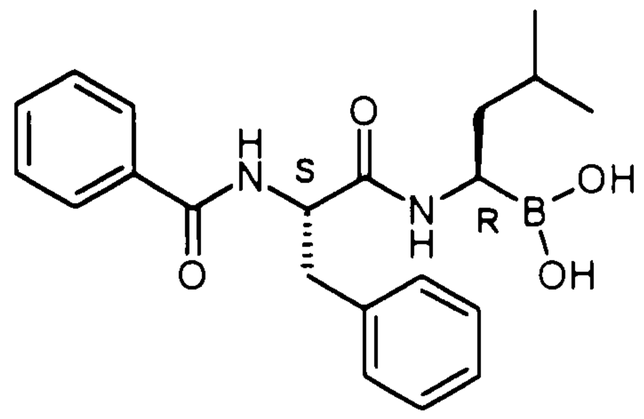
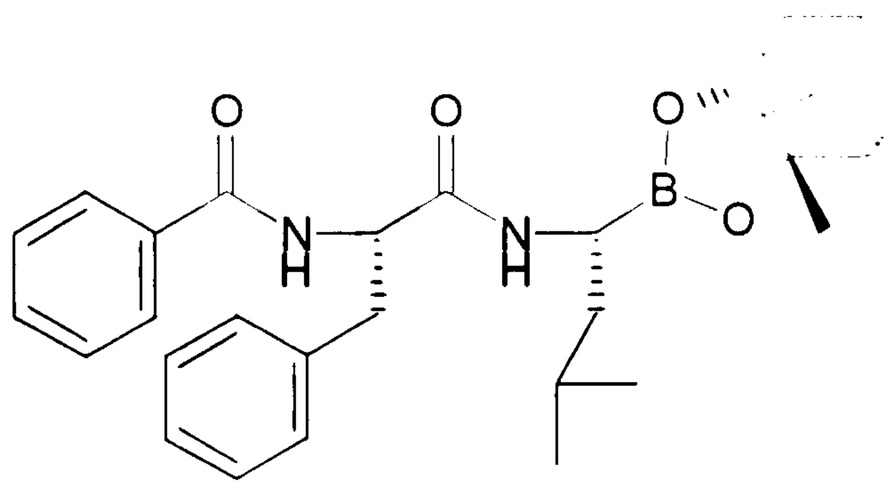
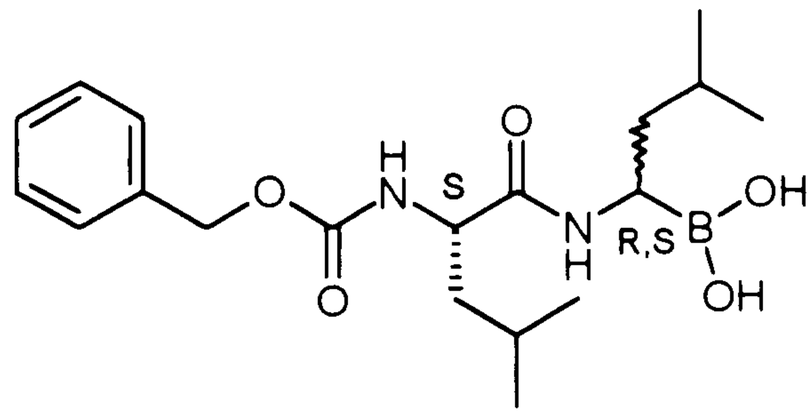
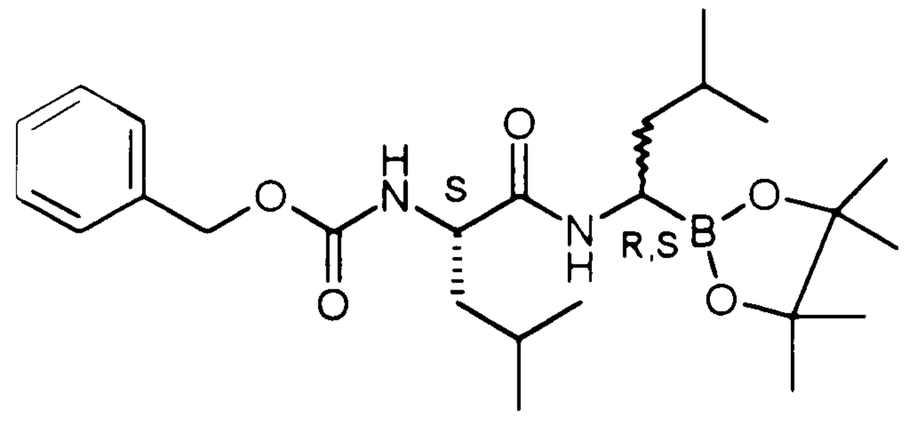
4.2 Determination of Ki values for the inhibition of the liver 20S proteasome LLVY activity by peptidyl boronic acids

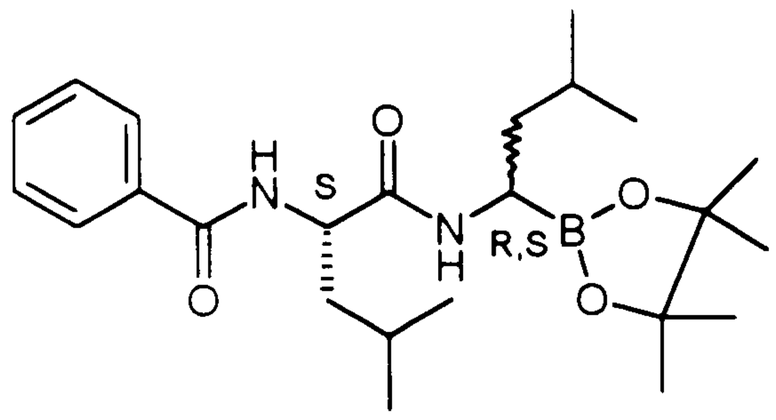
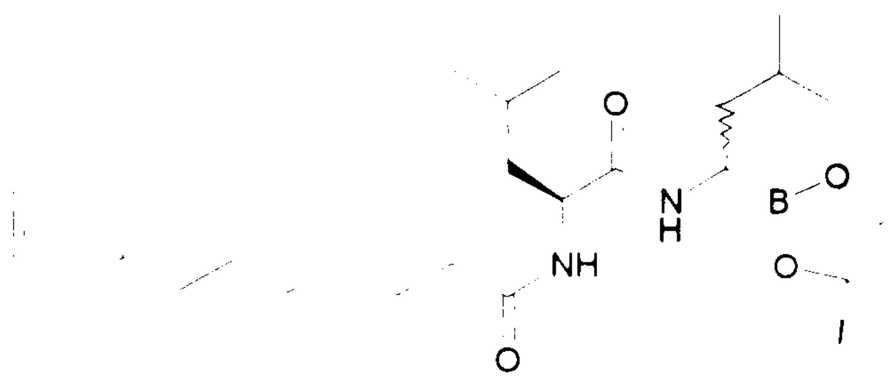
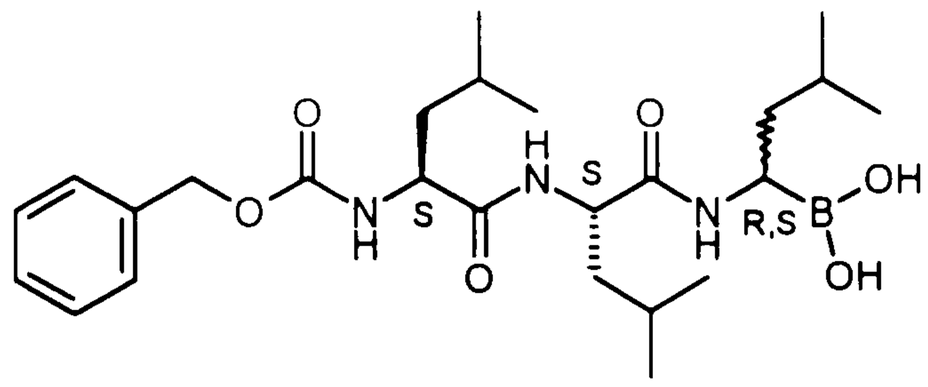
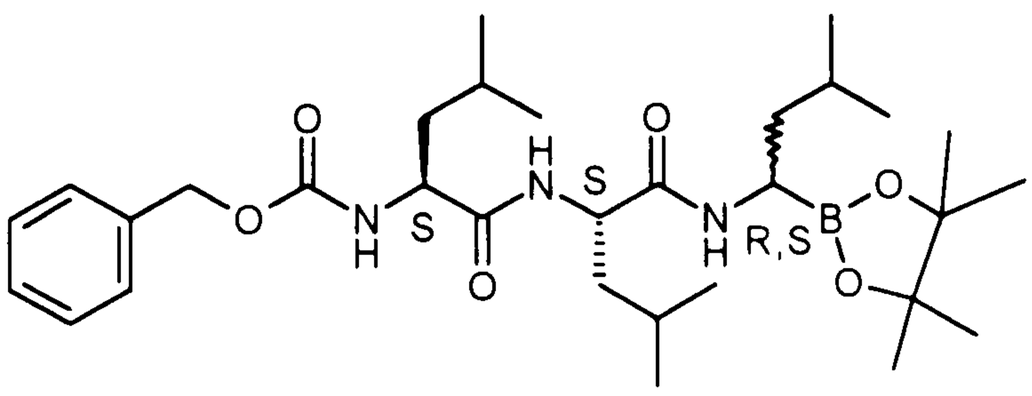
Ki values were determined using the competitive Henderson equation over a limited concentration range of 0 to 2×Ki. Ideal competitive tight-binding behaviour was observed from 0 to 2×Ki for all the tested peptidyl boronic acids. Deviation from ideal behaviour typically occurred at inhibitor concentrations above 2-3×Ki. Tables 4.1 and 4.2 list the structures and inhibitor constants for the peptidyl boronic acids. All the peptidyl boronic acids were synthesized with an N-terminal blocking group e.g. benzoyl or benzoyloxycarbonyl. If the N-terminus is unprotected the amide group can react with the boronic acid group to form an inactive cyclic structure (Coutts et al., 1996). As stated in chapter 3, the most effective

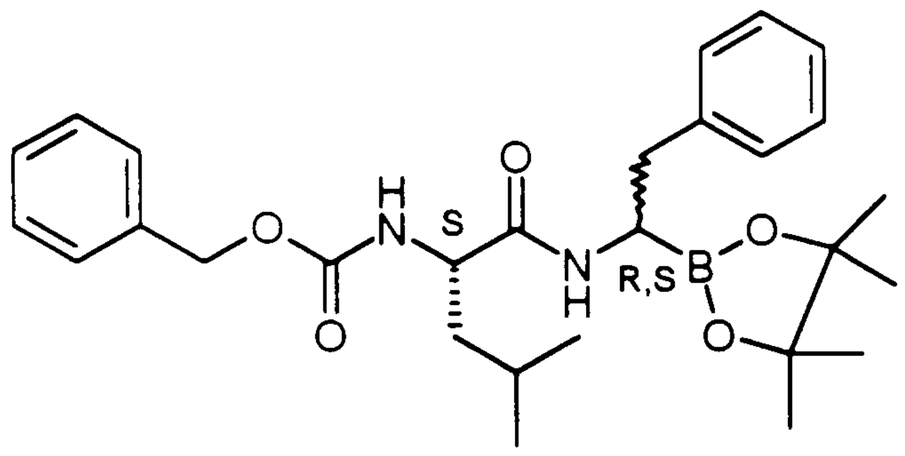
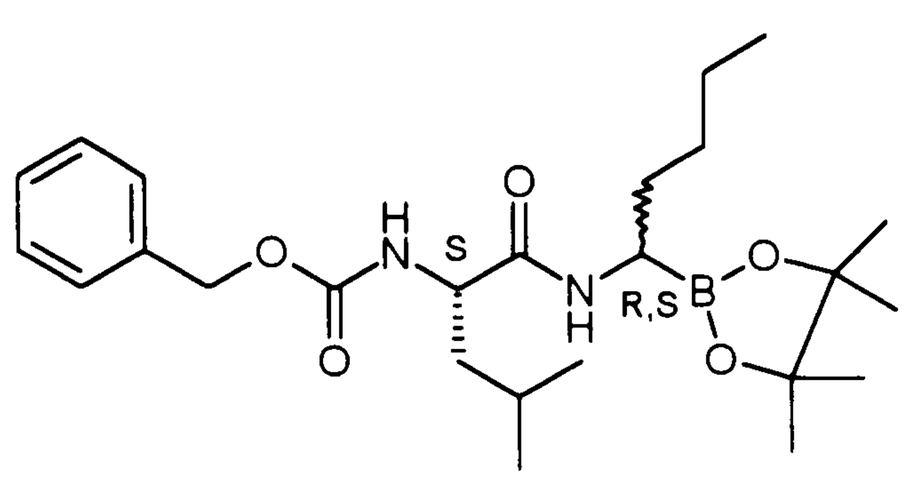
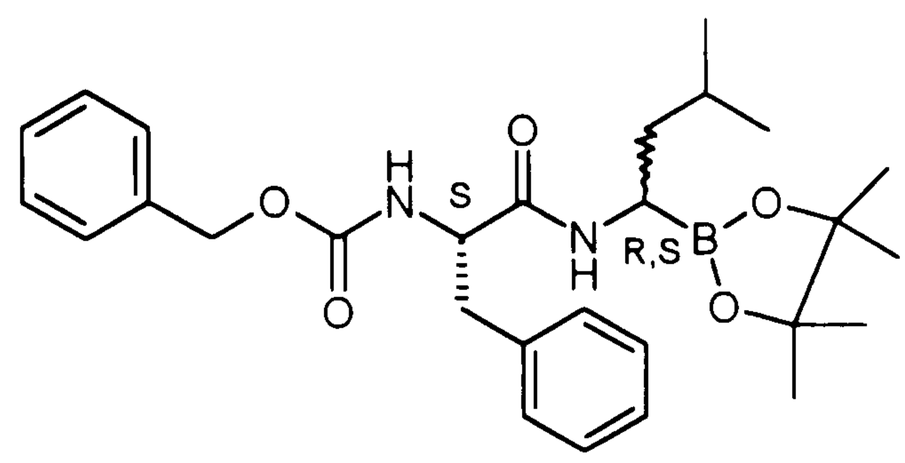
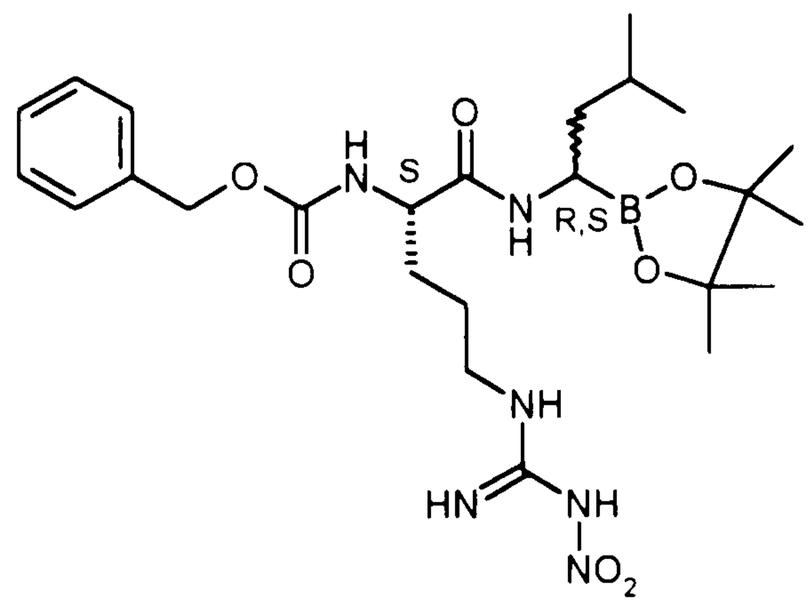
inhibitor of the liver 20S proteasome LLVY activity was Bz-Phe-boroLeu (compound 1). The pinane diol ester form of this inhibitor (compound 2) was less effective with a Ki of 53 nM. Hydrolysis of the hydrophobic pinane diol blocking group may be slow and incomplete in aqeuous solution, causing the observed reduction in Ki value (Coutts et al., 1996).

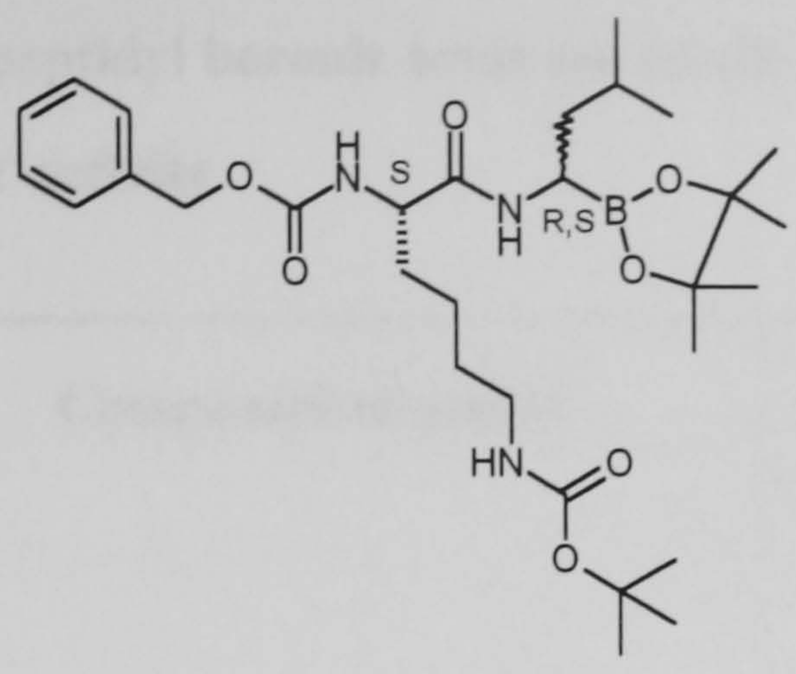
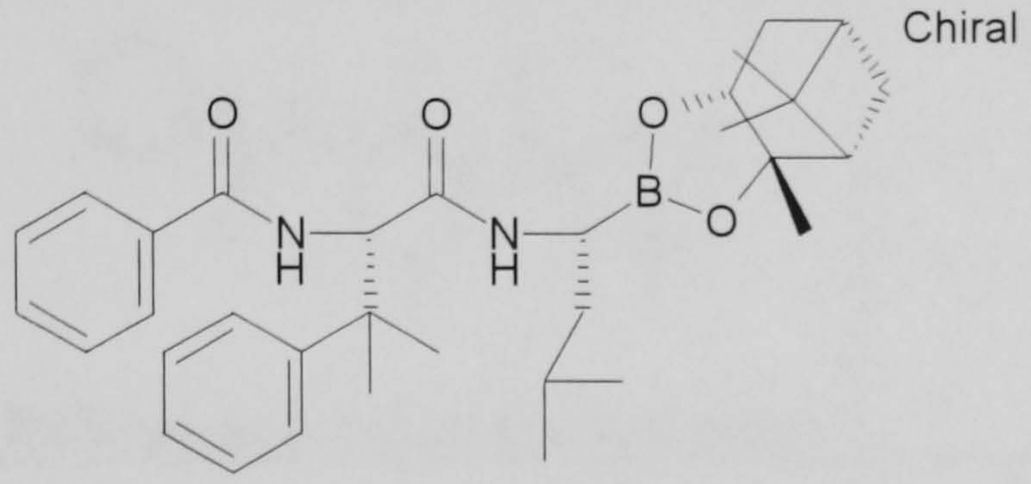
Dipeptidyl Leu-boroLeu based inhibitors were shown to be particularly potent. The Ki values for the free acid and pinacol ester forms of Cbz-Leu-boroLeu (compounds 3 and 4) were both in the region of 25-35 nM. This showed that the pinacol ester was readily hydrolysed from the Cbz-Leu-boroLeu structure during the assay procedure. Use of a benzoyl rather than a benzoyloxycarbonyl N-terminal group did not significantly change the potency of the Leu-boroLeu (pinacol ester) inhibitor (compare compounds 4 and 5). In contrast, Phenylsulfonyl-Leu-boroLeu (pinacol ester) (compound 15) was a relatively poor inhibitor causing 46% inhibition at 1 μM. This clearly indicates that the polar sulfonyl group is not easily accommodated in the inhibitor binding site. Phenylhexanyl-Leu-boroLeu (pinane diol ester) (compound 6) was a more effective inhibitor with a Ki of 116 nM. The elongated N-terminal group was not as well accommodated as Bz or Cbz groups.

Table 4.1 - Ki values for inhibition of the liver 20S proteasome LLVY activity by peptidyl boronic acids

Compound number	Compound structure	Concentration range (nM) and number of determinations	Ki value (nM)
1	 Bz-Phe-boroLeu (free acid)	0-40 (3)	17 ± 4
2	 Bz-Phe-boroLeu (pinane diol ester)	0-150 (4)	53 ± 5
3	 Cbz-Leu-boroLeu (free acid)	0-50 (3)	25 ± 10
4	 Cbz-Leu-boroLeu (pinacol ester)	0-60 (3)	35 ± 5

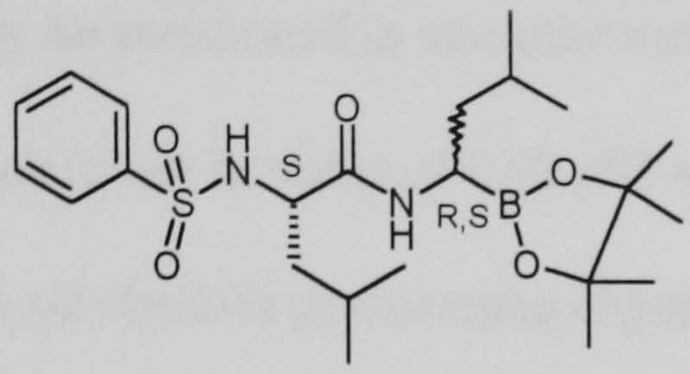
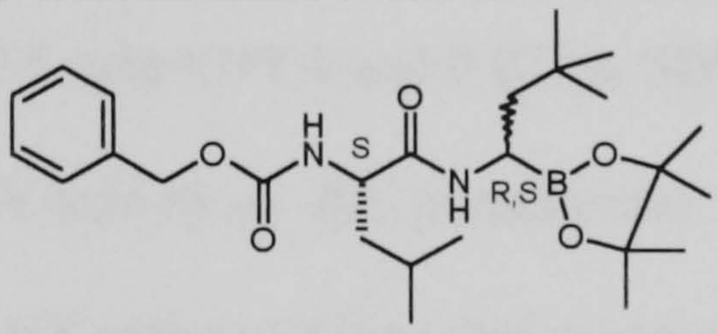
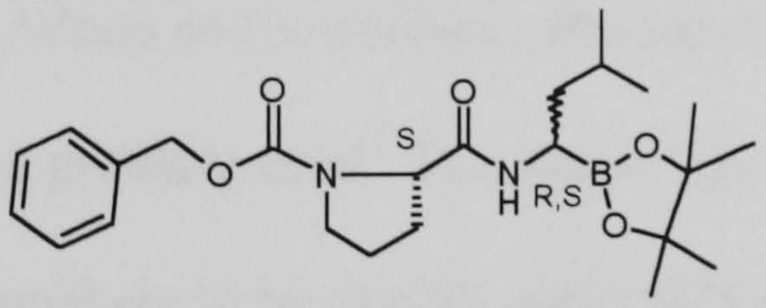
5	 Bz-Leu-boroLeu (pinacol ester)	0-50 (3)	23 ± 5
6	 Ph$(CH_2)_6$CO-Leu-boroLeu (pinacol ester)	0-150 (4)	116 ± 50
7	 Cbz-Leu-Leu-boroLeu (free acid)	0-100 (4)	46 ± 8
8	 Cbz-Leu-Leu-boroLeu (pinacol ester)	0-200 (4)	99 ± 19

9	 Cbz-Leu-boroPhe (pinacol ester)	0-200 (3)	84 ± 12
10	 Cbz-Leu-boroNle (pinacol ester)	0-200 (4)	97 ± 20
11	 Cbz-Phe-boroLeu (pinacol ester)	0-60 (3)	25 ± 11
12	 Cbz-nitroArg-boroLeu (pinacol ester)	0-100 (3)	39 ± 4

13	 Cbz-Lys(boc)-boroLeu (pinacol ester)	0-200 (3)	116 ± 10
14	 Bz-Val(Ph)-boroLeu (pinane diol ester)	0-500 (4)	201 ± 87

Assays were performed as described in Chapter 2 using 1 μg of liver 20S proteasome. The substrate concentration was 40 μM. Ki values were determined by fitting data from separate experiments performed in duplicate, to the competitive Henderson equation for tight-binding inhibitors. The curve fits were generated using the MicroMath Scientist 2.0 program. Ki values are given as the mean ± S.D. from three or four separate experiments.

Table 4.2 - Some peptidyl boronic acids are relatively weak inhibitors of the liver 20S proteasome LLVY activity

Compound number	Compound structure	Concentration range (μM)	LLVY activity (% of control) with 1 μM compound
15	 $PhSO_2$-Leu-boroLeu (pinacol ester)	0-1	54
16	 Cbz-Leu-t-butylboroLeu (pinacol ester)	0-1	73
17	 Cbz-Pro-boroLeu (pinacol ester)	0-1	91

Assays were performed as described in Chapter 2 using 1 μg of liver 20S proteasome. The substrate concentration was 40 μM. Values are given as the average of two separate experiments performed in duplicate

4.2.1 - Tri-leucine peptidyl boronic acids are less effective than di-leucine peptidyl boronic acids

Extending the amino acid structure by an additional P3 Leu residue did not increase the potency of the peptidyl boronic acids. Cbz-Leu-Leu-boroLeu and the corresponding pinacol ester (compounds 7 and 8) exhibited Ki values of 46 and 99 nM. The P3 Leu residue and/or the Cbz group may be positioned in an unfavourable location causing the observed reduction in potency. A much lower Ki value of 0.03 nM was previously reported for the effect of Cbz-Leu-Leu-boroLeu on the 20S proteasome chymotrypsin-like activity (Adams et al., 1998). The reasons for the large difference in Ki value are unclear. The assay buffer used by Adams et al. contained 0.5 mM EDTA and 0.035% SDS. This concentration of SDS is known to activate the LLVY activity of 20S proteasomes. Results in Figures 3.12 and 3.13 show that activating the LLVY activity of liver 20S proteasomes with 0.02% SDS, did not significantly change the Ki value of compound 1. The equation used to fit the experimental data was not clearly stated by Adams and coworkers. The equations describing parabolic inhibition in (Stein et al., 1996) were probably used. This model was successfully used with a non tight-binding inhibitor, but is unlikely to be directly applicable for a tight-binding inhibitor without some modification.

4.2.2 - Effect of changing either P1 or P2 residues of dipeptidyl boronic acids on the recorded Ki

The effect of altering P1 can be seen by comparing compounds 4, 9, 10 and 16. The isobutyl sidechain of leucine was best accommodated. The rank order of the other three P1 residues was Phe, Nle and t-butyl-Leu. It is interesting to note that the slightly more elongated n-butyl

structure of Nle, made a significant difference to the binding affinity of the boronic acid, compared to the iso-butyl structure of Leu. The bulky hydrophobic tert-butylmethyl side chain of t-butyl-Leu was least well accommodated. The effect of varying the P2 residue can be determined by comparing compounds 4, 11, 12, 13 and 17. Both compounds 4 and 11 had Ki values of 25-35 nM. Hence benzyl and isobutyl side chain are equally acceptable to the inhibitor binding site. A N^{γ}-nitroArg residue in P2 is also well accommodated (compound 12). The non-physiological N^{γ}-nitroArg residue is more elongated and polar than Phe or Leu. The structure of the inhibitor binding pocket may fold around this residue in a different manner to the more hydrophobic Phe and Leu residues. A related peptidyl boronic acid inhibitor of the 20S proteasome LLVY activity has been reported (Iqbal et al., 1996). This inhibitor had the same N^{γ}-nitroArg-boroLeu structure with a different N-terminal blocking group. The determined IC50 for this inhibitor was of a similar magnitude at 8 nM. Lys-(Boc) at P2 produces a relatively weak inhibitor of the LLVY activity (compound 13). The elongated sidechain ends in a tert-butyl group. This hydrophobic group is also found in compound 16 that was a very poor inhibitor of the LLVY activity. The least favourable P2 residue was Pro. Cbz-Pro-boroLeu (pinacol ester) was barely active at 1 μM concentration producing 9% inhibition. Proline is known to cause bending of polypeptide chains. Hence the Pro residue probably causes the inhibitor structure to kink creating unfavourable interactions with nearby protein residues. Proline has been used successfully in the construction of peptidyl boronic acid inhibitors of other enzymes. Dipeptidyl peptidase IV is a serine protease that cleaves a dipeptide from the amino terminus of polypeptides, where the penultimate residue is proline. A series of highly effective inhibitors of the general structure H_2N-X_{aa}-boroPro were synthesized (Coutts et al., 1996). Bz-Val(Ph)-boroLeu (pinane diol ester) was structurally related to compounds 4, 11, 12, 13, and 17. This was one of the least effective inhibitors with

a Ki of 201 nM.

4.2.3 - The trypsin-like activity of liver 20S proteasomes can be inhibited using high concentrations of peptidyl boronic acids

In a series of experiments, the effect of selected peptidyl boronic acids on the liver 20S proteasome LSTR activity was investigated. The assays were conducted using 2 μg portions of liver 20S proteasome, 40 μM Boc-LSTR-AMC and 0-1 μM peptidyl boronic acid. A number of the peptidyl boronic acids did not inhibit the LSTR activity at 1 μM concentration. These included Bz-Val(Ph)-boroLeu (pinane diol ester), $PhSO_2$-Leu-boroLeu (pinacol ester), Cbz-Leu-t-butylboroLeu (pinacol ester) and Cbz-Pro-boroLeu (pinacol ester). All four of these peptidyl boronic acids were relatively poor inhibitors of the liver 20S proteasome LLVY activity (Tables 4.1 and 4.2).

Table 4.3 illustrates the extent of inhibition produced by the more effective inhibitors of the LSTR activity. Both the free acid and pinacol ester forms of Cbz-Leu-Leu-boroLeu were relatively potent inhibitors of the LSTR activity. This was intriguing because both Leu-Leu-Leu-Leu-vinyl sulphone and Bz-Phe-Leu-Leu-Leu-vinyl sulphone were relatively potent inhibitors of the muscle 20S proteasome trypsin-like activity (Bogyo et al., 1998). The importance of a large P4 residue was demonstrated by the poor inhibitory effect of Leu-Leu-Leu-vinyl sulphone and Gly-Leu-Leu-Leu-vinyl sulphone. The moderate activity of Tyr-Leu-Leu-Leu-vinyl sulphone confirmed that the structure of the bulky P4 residue was also important. 1 μM Bz-Phe-boroLeu (compound 1) caused inhibition of the LSTR activity. This effect was found to be dependent on the 20S proteasome concentration. When the 20S

proteasome concentration was lowered from 0.01 to 0.005 mg/ml the same level of inhibition was observed. Increasing the 20S proteasome concentration from 0.01 to 0.02 mg/ml caused 1 μM compound 1 to moderately stimulate the LSTR activity (see Table 3.4). The reasons for these observations were unclear. Modification of trypsin-like catalytic sites may only take place at permissable [20S proteasome]:[compound 1] ratios. 1 μM Cbz-Leu-Leu-boroLeu (pinacol ester) was shown to be equally effective at 20S proteasome concentrations of 0.01 and 0.02 mg/ml (Tables 4.3 and 3.4).

Table 4.3 - Tri-leucine peptidyl boronic acids are the most potent inhibitors of the liver 20S proteasome trypsin-like activity

Peptidyl boronic acid (compound no.)	100 nM (% of control)	1 μM (% of control)
Bz-Phe-boroLeu (compound 1)	122	64
Cbz-Leu-Leu-boroLeu (compound 7)	100	48
Cbz-Leu-Leu-boroLeu (pinacol ester) (compound 8)	97	56
Cbz-Leu-boroPhe (pinacol ester) (compound 9)	101	80
Cbz-nitroArg-boroLeu (pinacol ester) (compound 12)	102, 103	81, 85
Cbz-Lys(boc)-boroLeu (pinacol ester) (compound 13)	101, 102	70, 70

Assays were performed as described in Chapter 2 using 2 μg of liver 20S proteasome. The Boc-LSTR-AMC concentration was 40 μM. Values are from one or two experiments performed in duplicate. These values are expressed as percent of control activity in samples containing no inhibitor.

4.3 - SDS-PAGE analysis of liver and spleen 20S proteasomes

Figure 4.1 shows a SDS-PAGE separation of 20S proteasomes purified from rat spleen and liver. The gel was scanned using a Bio-Rad GS-90 densitometer and Molecular Analyst software supplied by Bio-Rad. Note the characteristic pattern of subunits with molecular weights of 20-34 kDa. A small amount of high molecular weight (~ 90 kDa) impurity protein was observed in the rat spleen lane. Densitometric analysis of this lane was conducted using the Molecular Analyst software. The amount of impurity protein was judged to be less than 2% of the total protein. This 90 kDa protein is sometimes observed in rat liver 20S proteasome preparations and may be HSP90.

4.3.1 - Determination of the Km for hydrolysis of Suc-LLVY-AMC by spleen 20S proteasomes

The Km for Suc-LLVY-AMC hydrolysis by 1 μg spleen 20S proteasome was determined as described in Chapter 2. Figure 4.2 shows a typical set of data fitted to the Michaelis-Menten equation. The average Km was 63 ± 8 μM from three determinations. This value was approximately half the value of 130 μM, determined for the LLVY activity of liver 20S proteasomes. The value of Vmax was also reduced by approximately half. Therefore the k_{cat}/Km ratio or catalytic efficiency of liver and spleen 20S proteasomes are approximately equal. Spleen 20S proteasomes bind more efficiently to the Suc-LLVY-AMC substrate but are less effective at catalysing the hydrolysis of the substrate.

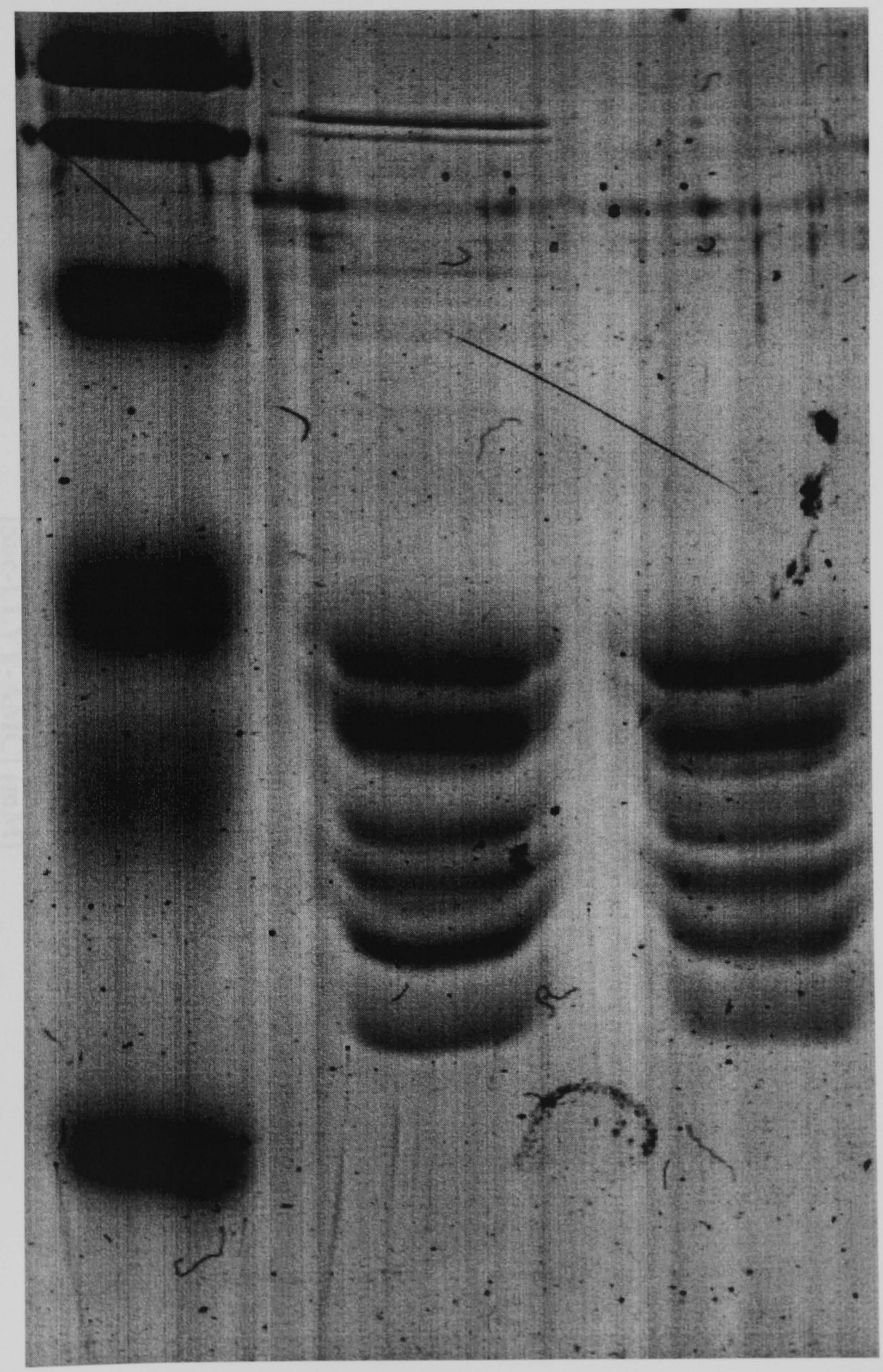
Figure 4.1 - SDS-PAGE analysis of rat spleen and rat liver 20S proteasome (page 132)

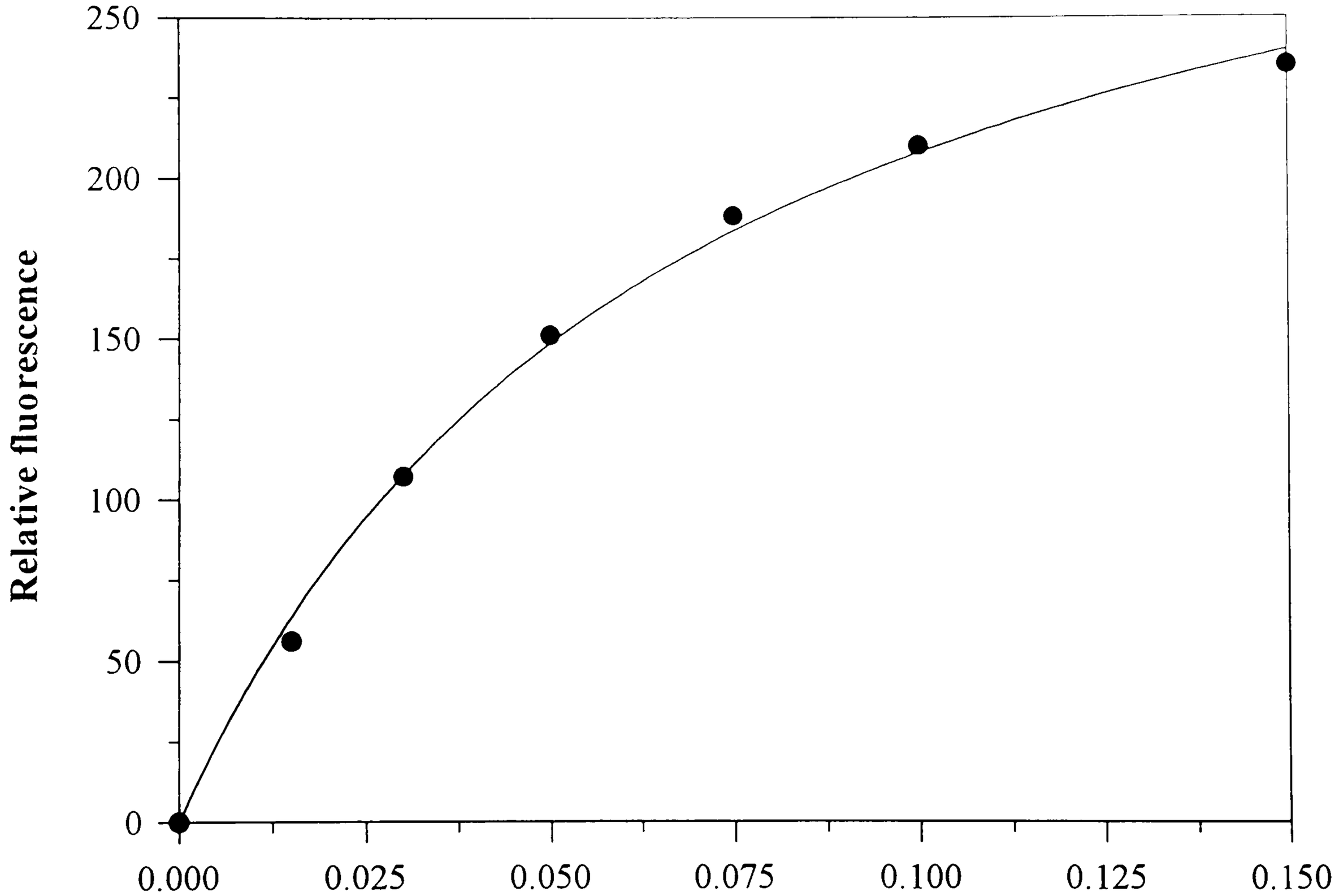
Spleen and liver 20S proteasomes (4 μg/sample) were prepared and run on a 15% SDS-PAGE gel as described in Chapter 2. The gel was stained with Coomassie brillant blue stain solution and then destained as described in Chapter 2. The destained gel was scanned using a Bio-Rad GS-90 densitometer and Molecular Analyst software supplied by Bio-Rad. The three lanes are labelled M (marker protein lane), S (spleen 20S proteasome) and L (liver 20S proteasome).

Figure 4.2 - Hydrolysis of Suc-LLVY-AMC by spleen 20S proteasomes (page 133)

Spleen 20S proteasome (1 μg) was assayed against a range of Suc-LLVY-AMC concentrations in 50 mM Hepes buffer/KOH, pH 7.5. Assays were conducted for 15 minutes at 37°C, as described in Chapter 2. Results from a typical experiment performed in duplicate are shown. Km values were determined by fitting the data from separate experiments to the Michaelis-Menten equation. The average Km was 63 ± 8 μM from three separate determinations.

M S L




250
200
150
100
50
0
0.000
0.025
0.050
0.075
0.100
0.125
0.150
Relative fluorescence
[Suc-LLVY-AMC] (mM)

4.4 - Liver and spleen 20S proteasomes display different specific activities to trypsin-like and PGPH substrates

Table 4.4 compares the specific activities of liver and spleen 20S proteasomes to four peptidase substrates. The specific activity of liver 20S proteasomes to the Suc-LLVY-AMC and AAF-AMC substrates were shown to be similar and in line with previous liver 20S proteasome preparations (Rivett et al., 1994). Cbz-GGL-AMC was less effectively cleaved by the liver 20S proteasome. One study investigated how the nature of the amino acids affected the hydrolysis of larger tetra- and pentapeptide substrates. The nature of the P1 and P3 amino acids, was proposed to influence the cleavage of a substrate by a particular catalytic site (Cardozo et al., 1994). The specific activities for hydrolysis of the three chymotrypsin-like substrates by spleen 20S proteasomes were comparable to liver 20S proteasomes. In contrast the specific activity of spleen 20S proteasomes to Boc-LSTR-AMC was three times lower than liver 20S proteasomes. The β-subunits Z and MECL1 have been proposed to catalyse the trypsin-like activity (Bogyo et al., 1998). This result suggests that the catalytic efficiency of MECL1 is much less than Z. Eleuteri and coworkers (1997) found only a small difference in the specific activity of spleen and pituitary 20S proteasomes, measured using Cbz-(D)ALR-NAP. Pituitary 20S proteasomes contain very low levels of IFN-γ-inducible β-subunits. However, these experiments were performed with 10-fold more substrate than the results described in Table 4.4. This higher substrate concentration produced a 16-fold increase in activity. Therefore at the higher substrate concentration both catalytic sites may be equally efficient.

Table 4.4 - Comparison of the specific peptidase activities of liver and spleen 20S proteasomes

Substrate	Liver 20S proteasome specific activity (nmol/min/mg protein)	Spleen 20S proteasome specific activity (nmol/min/mg protein)
Suc-LLVY-AMC	10 ± 1	12
AAF-AMC	9 ± 1	9
Cbz-GGL-AMC	5 ± 0	5
Boc-LSTR-AMC	5 ± 1	2

Assays were performed as described in Chapter 2 using 1 μg of liver or spleen 20S proteasome. The substrate concentration was 40 μM for Suc-LLVY-AMC, AAF-AMC, Cbz-GGL-AMC and Boc-LSTR-AMC. A fluorescence standard curve for 7-amino-4-methylcoumarin (AMC) quantities of 0-0.12 nmol was produced as described in Chapter 2. The equation for the standard curve was then used to calculate the specific activities. Values for the liver 20S proteasome were from two separate experiments performed in duplicate. The range of the readings are listed. Values for the spleen 20S proteasome were from one experiment performed in duplicate.

A fluorescence standard curve for β-naphthylamide was not produced. Fluorescence readings obtained from assays using 1 μg liver 20S proteasome and either 40 μM Suc-LLVY-AMC or 100 μM Cbz-LLE-NAP were compared. This comparison suggested that the LLE1 activity was ~ 10 times less than the LLVY activity. Of note was the fact that the LLE1 activity of 1 μg liver 20S proteasome was three times greater than 1 μg spleen 20S proteasome. Eleuteri et al. (1997) showed that the PGPH activity of spleen 20S proteasomes was eight times lower than pituitary 20S proteasomes. In another study 20S proteasomes were purified from U937 cells either cultured in the absence or presence of human IFN-γ. IFN-γ treatment caused a small (13%) decrease in the PGPH activity (Gaczynska et al., 1993). Proteasome complexes are proposed to play an important role in generating antigenic peptides for MHC class I molecules. These peptides end almost exclusively with C-terminal hydrophobic or basic residues. Therefore a reduction in the PGPH activity will cause more of the required peptides to be produced, assisting the immune response (Gaczynska et al., 1993; Benham & Neefjes, 1997).

4.5 - Time course for inhibition of the spleen 20S proteasome LLVY activity by peptidyl boronic acids is consistent with slow binding

Figure 4.3A (page 139) shows the time course for inhibition of the spleen 20S proteasome LLVY activity by 10 nM compound 1. In the presence of 10 nM compound 1 the fully inhibited rate of reaction was only observed after approximately 10-12 minutes. Studies using radiolabelled vinyl sulphone inhibitors suggested that subunits X/MB1 and LMP7 were predominantly responsible for the chymotrypsin-like activity of 20S proteasomes (Table 1.3) (Bogyo et al., 1998). The results of Reidlinger and coworkers (1997) using [^{3}H]-acetyl-Ala-

Ala-Phe-CH_2Cl supported this assignment. In contrast, studies using [^{14}C] labelled 3,4-dichloroisocoumarin and peptidyl aldehydes suggested that subunit X/MB1 of pituitary 20S proteasomes and LMP2 of spleen 20S proteasomes catalysed the chymotrypsin-like activity (Orlowski et al., 1997). Whatever the final assignment of catalytic subunits to the chymotrypsin-like activity, these catalytic subunits are inhibited by peptidyl boronic acids through an apparent slow-binding mechanism.

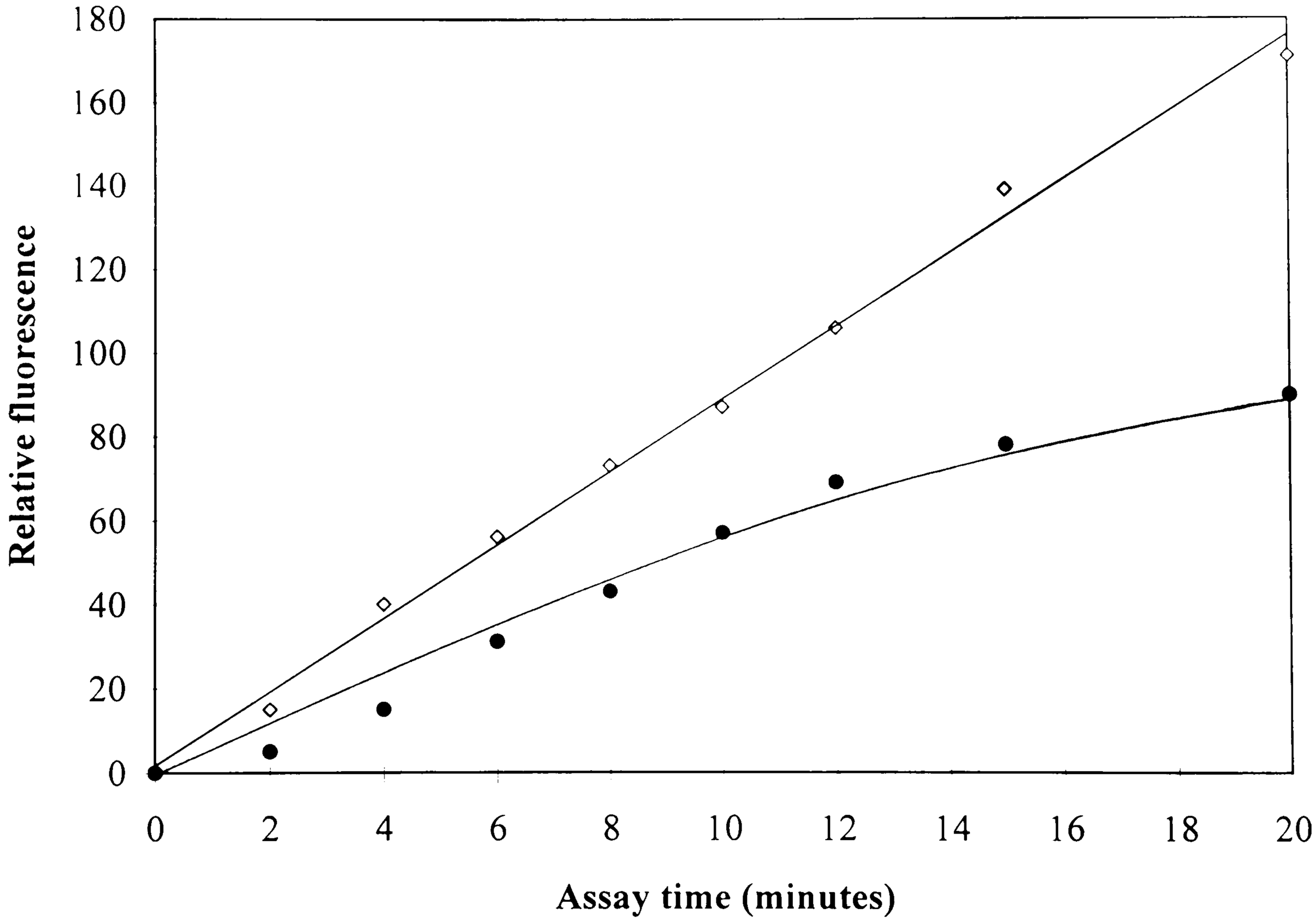
4.5.1 - Apparent slow binding is also observed at high Bz-Phe-boroLeu (compound 1) concentrations

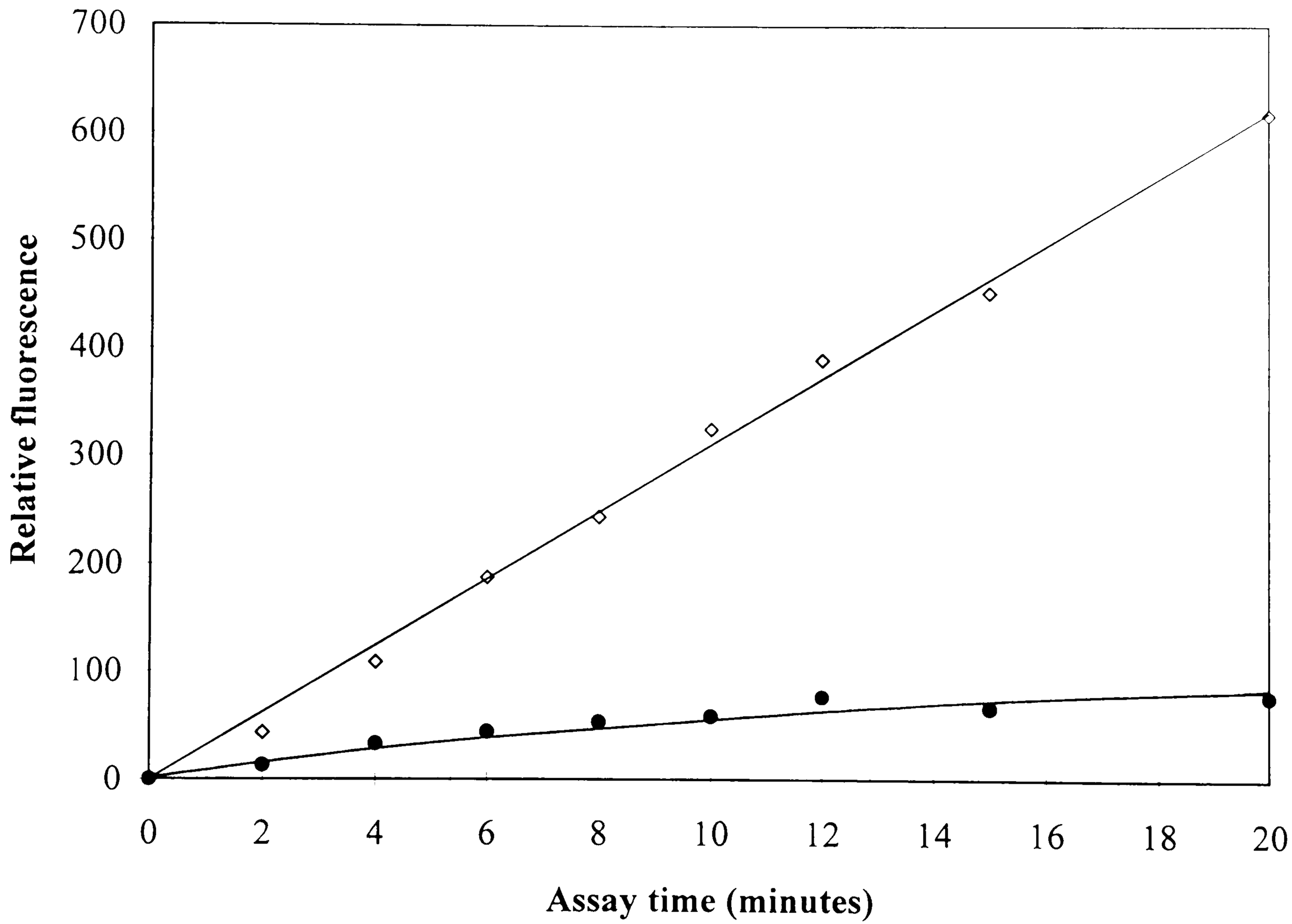
Figure 4.3B (page 140) shows the time course for inhibition of the spleen 20S proteasome LLVY activity by 100 nM compound 1. The Ki for compound 1 against the LLVY activity of 1 μg spleen 20S proteasome was determined to be 13 nM (Table 4.6). Therefore the compound 1 concentration used in this experiment was eight times higher than Ki. At this concentration the full inhibitory effect was observed after a shorter time period of 5-7 minutes. This result suggests that steric hindrance probably plays a significant role in apparently slowing the onset of full inhibition, by 10 nM compound 1. Use of a large amount of compound 1 will tend to overcome any hindrance of inhibitor movement towards the catalytic sites and increase the concentration of inhibitor in close proximity to the catalytic sites. Both of these factors probably decrease the time required for covalent modification to occur.

Figure 4.3 - Time course for inhibition of the spleen 20S proteasome LLVY activity by 10 or 100 nM Bz-Phe-boroLeu (compound 1)

Spleen 20S proteasome (1 μg) was incubated at 37°C in the presence (filled circles) or absence (open diamonds) of 10 nM (Panel A) or 100 nM (Panel B) Bz-Phe-boroLeu. Substrate (40 μM Suc-LLVY-AMC) was present from time zero and substrate hydrolysis was determined after incubation for selected times as described in Chapter 2.

Panel A on page 139. Panel B on page 140.


Relative fluorescence
180
160
140
120
100
80
60
40
20
0
0
2
4
6
8
10
12
14
16
18
20
Assay time (minutes)


700
600
500
400
300
200
100
0
Relative fluorescence
0
2
4
6
8
10
12
14
16
18
20
Assay time (minutes)

4.6 - Compound 1 (80 nM) inhibits the LLVY activity but not the LLE1 activity of spleen 20S proteasomes

Table 4.5 illustrates the effect of 20 and 80 nM compound 1 on the LLVY, AAF, GGL, LSTR and LLE1 activities of spleen 20S proteasomes. At the selected concentrations of compound 1 only the LLVY activity was significantly inhibited. The results for the PGPH activity are in contrast to liver 20S proteasomes. The liver 20S proteasome LSTR activity was inhibited 39% by 80 nM compound 1. Studies using radiolabelled peptidyl vinyl sulphones linked inhibition of the PGPH activity with modification of subunits LMP2 and Y/δ (Table 1.3) (Bogyo et al., 1998). A separate study using [^{14}C]-3,4-dichloroisocoumarin also linked the PGPH activity to subunit Y/δ (Orlowski et al., 1997). If LMP2 and Y/δ are predominantly responsible for the PGPH activity, modification of Y/δ but not LMP2 at 80 nM compound 1 would explain these results. As discussed previously, stimulation of the trypsin-like and PGPH activities may result from conformational changes caused by inhibitor modification of the chymotrypsin-like catalytic sites.

Table 4.5 - Bz-Phe-boroLeu (compound 1) (80 nM) inhibits the LLVY activity and stimulates the trypsin-like and PGPH activities of spleen 20S proteasomes

Substrate	Activity (% of control) 20 nM compound 1	Activity (% of control) 80 nM compound 1
Suc-LLVY-AMC	52	34
AAF-AMC	95	86
Cbz-GGL-AMC	96	87
Boc-LSTR-AMC	107	108
Cbz-LLE-NAP	108	121

Assays were performed as described in Chapter 2 using 1 μg of spleen 20S proteasome. The substrate concentration was 40 μM for Suc-LLVY-AMC, AAF-AMC, Cbz-GGL-AMC and Boc-LSTR-AMC. Cbz-LLE-NAP was used at 100 μM. Values are from one experiment performed in duplicate. These values are expressed as percent of control activity in samples containing no inhibitor.

4.7 - Compound 1 inhibits the LLVY activity of liver and spleen 20S proteasomes in a very similar manner

Figure 4.4 shows the effect of selected concentrations of compound 1 on the spleen 20S proteasome LLVY activity. The competitive Henderson equation was used to generate the curve fit to the data. The effect of compound 1 on the LLVY activity of 1 μg spleen 20S proteasome was very similar to the corresponding experiment for liver 20S proteasomes shown in Figure 3.3. The curve fit in both figures followed the velocity data over a limited [compound 1] range. Deviation from ideal behaviour was found to occur at approximately 70 nM for both types of 20S proteasome. Figure 4.5 shows the effect of selected concentrations of Cbz-Leu-Leu-boroLeu (compound 7) on the spleen 20S proteasome LLVY activity. Of interest is the comparison between Figures 4.5 and 3.4. Inhibition of the spleen 20S proteasome LLVY activity by compound 7 follows ideal behaviour up to 200 nM. This was equivalent to 5×Ki. In Figure 3.4 inhibition of the liver 20S proteasome LLVY activity by compound 7, deviated from ideal behaviour above 100 nM (2×Ki). Furthermore the deviation from ideal behaviour was greater for the liver 20S proteasome compared to the spleen 20S proteasome, particularly at 0.5 and 1 μM. The tri-Leu arrangement of compound 7 may cause greater conformational changes than the Phe-Leu arrangement of compound 1. This could cause an increase in Suc-LLVY-AMC hydrolysis at the trypsin-like and PGPH catalytic sites in liver 20S proteasomes. When compared to liver 20S proteasomes, spleen 20S proteasomes exhibit three-fold lower specific activities to trypsin-like and PGPH substrates. Hence the basal level of LLVY activity at these sites may be lower. Modification of LLVY hydrolysing sites may stimulate little or no increase in LLVY hydrolysis at other sites, thus explaining the greater adherence to ideal behaviour. In support of this theory was the observation that

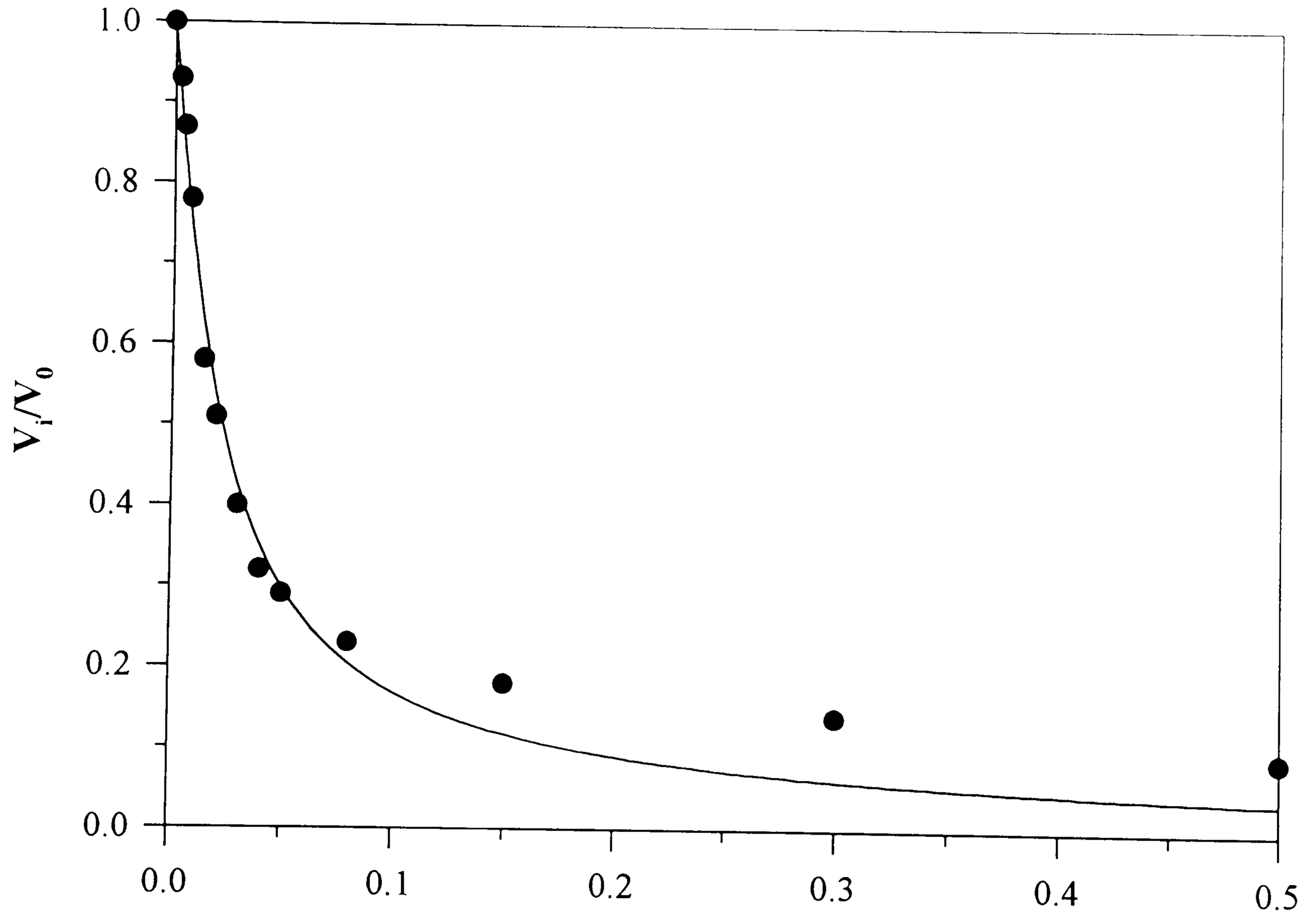
inhibition of 1 μg liver 20S proteasome by the large Cbz-nitroArg-boroLeu (pinacol ester) inhibitor, significantly deviated from ideal behaviour at high inhibitor concentrations (data not shown). Unfortunately this inhibitor was not tested with the spleen 20S proteasome.

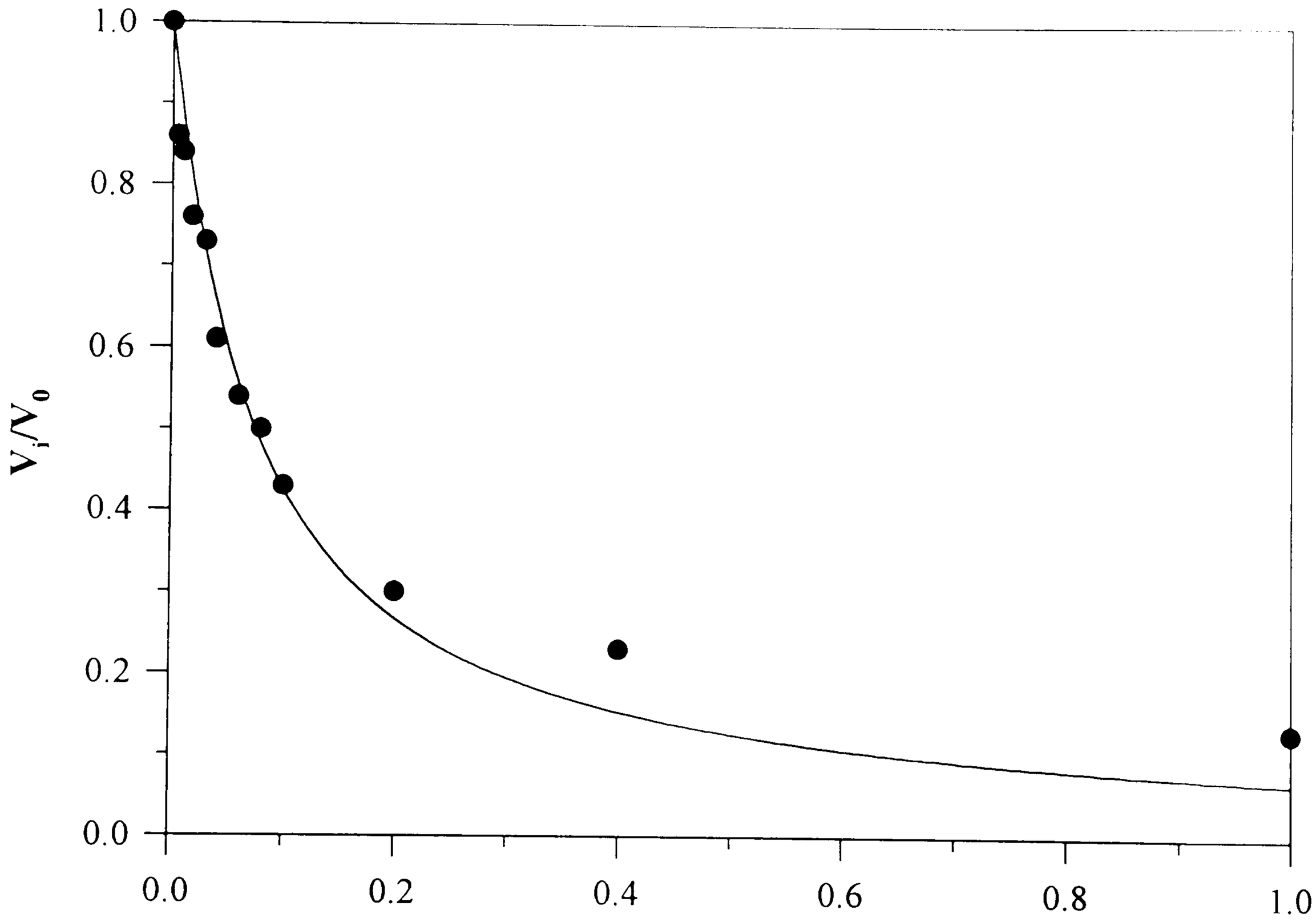
Figure 4.4 - Inhibition of the spleen 20S proteasome LLVY activity by Bz-Phe-boroLeu (compound 1) (page 145)

Spleen 20S proteasome (1 μg) was incubated with selected concentrations of compound 1 in 50 mM Hepes buffer/KOH, pH 7.5, for 15 minutes at 25°C. Substrate (40 μM Suc-LLVY-AMC) was added and the assays were incubated at 37°C for 15 minutes, as described in Chapter 2. Values are from a typical experiment performed in duplicate. Activities are expressed as the fraction of control activity in samples containing no inhibitor. The competitive Henderson equation for tight-binding inhibitors was used to generate a curve fit to the data.

Figure 4.5 - Inhibition of the spleen 20S proteasome LLVY activity by Cbz-Leu-Leu-boroLeu (compound 7) (page 146)

Experiments were performed as described in the legend to Figure 4.4 using selected concentrations of Cbz-Leu-Leu-boroLeu.


1.0
0.8
0.6
0.4
0.2
0.0
V_i/V_0
0.0
0.1
0.2
0.3
0.4
0.5
[Bz-Phe-boroLeu] (μM)


1.0
0.8
0.6
0.4
0.2
0.0
V_i/V_0
0.0
0.2
0.4
0.6
0.8
1.0
[Cbz-Leu-Leu-boroLeu] (μM)

4.8 - Ki values for inhibiting the LLVY activity of liver and spleen 20S proteasomes are very similar

Table 4.6 compares the Ki values for inhibition of the liver and spleen 20S proteasome LLVY activity by peptidyl boronic acids. Five peptidyl boronic acids were chosen to investigate whether the Ki values for the spleen 20S proteasome would differ significantly from the liver 20S proteasome. These included the most effective inhibitor of the liver 20S proteasome LLVY activity, compound 1 and the closely related compound Cbz-Phe-boroLeu (pinacol ester). Two of the less effective dipeptidyl boronic acids, Cbz-Leu-boroPhe (pinacol ester) and Bz-Val(Ph)-boroLeu (pinane diol ester) were chosen along with the most effective tripeptidyl boronic acid Cbz-Leu-Leu-boroLeu. Large differences in the Ki values were not observed. Compared to liver 20S proteasomes the Ki for Cbz-Phe-boroLeu (pinacol ester) and Bz-Val(Ph)-boroLeu (pinane diol ester) using spleen 20S proteasomes increased 1.9 and 1.5 times respectively. In contrast the Ki values for Bz-Phe-boroLeu and Cbz-Leu-Leu-boroLeu using spleen 20S proteasomes decreased 1.4 and 1.3 fold respectively. The β-subunits X/MB1 and LMP7 are proposed to catalyse the chymotrypsin-like activity of mammalian proteasomes (Table 1.3). Approximately equal amounts of X/MB1 and LMP7 are found in liver 20S proteasomes, whereas spleen 20S proteasomes contain less than 10% X/MB1 compared to LMP7 (Eleuteri et al., 1997; P.Brooks, personal communication). Therefore the observed differences may reflect the different affinities of the peptidyl boronic acids for the two different catalytic sites. X/MB1 and LMP7 exhibit a high degree of identity. However, single residue changes at or close to the inhibitor binding site may favour or obstruct peptidyl boronic acid binding.

Table 4.6 - Peptidyl boronic acids inhibit the LLVY activity of liver and spleen 20S proteasomes

Peptidyl boronic acid (compound no.)	LLVY activity			
	Liver 20S proteasome		Spleen 20S proteasome	
	Concentration range (nM) and number of determinations	Ki value (nM)	Concentration range (nM) and number of determinations	Ki value (nM)
Bz-Phe-boroLeu (compound 1)	0-40 (3)	17 ± 4	0-30 (2)	13 (range ± 2)
Cbz-Phe-boroLeu (pinacol ester) (compound 11)	0-60 (3)	25 ± 11	0-100 (2)	45 (range ± 7)
Cbz-Leu-Leu-boroLeu (compound 7)	0-100 (4)	46 ± 8	0-80 (2)	35 (range ± 5)
Cbz-Leu-boroPhe (pinacol ester) (compound 9)	0-200 (3)	84 ± 12	0-210 (2)	92 (range ± 0)
Bz-Val(Ph)-boroLeu (pinane diol ester) (compound 14)	0-500 (4)	201 ± 87	0-650 (2)	307 (range ± 1)

Assays were conducted as described in Chapter 2 using 1 μg of either liver or spleen 20S proteasome. Values are from 2-4 separate experiments performed in duplicate. Ki values were determined by fitting data from separate experiments to the competitive Henderson equation.

4.9 - Summary

Bz-Phe-boroLeu was the most effective of the seventeen peptidyl boronic acids, tested against the liver 20S proteasome LLVY activity. Other dipeptidyl boronic acids e.g. Cbz-Leu-boroLeu and Bz-Leu-boroLeu (pinacol ester) were also particularly effective inhibitors of this activity. Tri-leucine compounds e.g. Cbz-Leu-Leu-boroLeu were less effective, although these compounds were the best inhibitors of the trypsin-like activity. Compounds containing Pro at P2 or large bulky hydrophobic groups at P1/P2 e.g. Lys(boc), Val(Ph) or t-butylLeu were relatively ineffective. In contrast Cbz-nitroArg-boroLeu (pinacol ester) containing the large polar nitroArg residue, was one of the most effective inhibitors. Pinacol ester blocked peptidyl boronic acids were generally as effective as the corresponding unblocked compounds. Results obtained with Bz-Phe-boroLeu and Bz-Phe-boroLeu (pinane diol ester) suggested that the pinane diol group is only partially hydrolysed during the assay procedure. The specific activity of liver or spleen 20S proteasomes to chymotrypsin-like substrates was approximately equal. In contrast the specific activity of spleen 20S proteasomes to trypsin-like or PGPH substrates was three-fold lower than liver 20S proteasomes. These results are in line with the proposal that proteasomes containing the IFN-γ inducible catalytic subunits, play a major role in producing antigenic peptides that exhibit hydrophobic or basic C-terminal residues. Bz-Phe-boroLeu was a tight-binding inhibitor of the spleen 20S proteasome LLVY activity, with inhibition apparently occurring through a slow binding mechanism. Ki values for inhibition of the liver or spleen 20S proteasome LLVY activity by selected peptidyl boronic acids, were shown to be very similar.

Chapter 5 - Inhibition of proteasome complexes in cultured cells and radiolabelling of proteasomes in vivo and in vitro

Chapter 5 - Inhibition of proteasome complexes in cultured cells and radiolabelling of proteasomes in vivo and in vitro

5.1 - Introduction

As described in Chapter 1, four separate proteasome complexes are observed inside eukaryotic cells. These are named the 26S proteasome, the 20S proteasome, the PA28-20S proteasome complex and the hybrid proteasome. The 26S proteasome catalyses ubiquitin-dependent and ubiquitin-independent pathways of protein degradation. Ubiquitinated substrates of the 26S proteasome include the transcriptional activators Gcn4, STAT1 and c-Jun. Other ubiquitinated substrates include a number of cell cycle regulatory proteins e.g. c-Mos and various cyclins. Modulators of immune and inflammatory responses are also ubiquitinated and degraded e.g. IκBα or partially degraded e.g. during the production of the mature NF-κB p50 subunit (Rivett et al., 1995; Kuehn & Dahlmann, 1996; Hilt & Wolf, 1996; Kim & Maniatis, 1996; Ishida et al., 1993; Alkalay et al., 1995; Palombella et al., 1994, Lin et al. 1998b). Proteasome complexes are also implicated in the generation of antigenic peptides, for presentation on class I MHC molecules (Grant et al., 1995; Hughes et al., 1996; Vinitsky et al., 1997). Potent cell permeable inhibitors of proteasome complexes, are valuble tools for investigating the degradation of proteins and the biochemistry of various diseases.

In this chapter I will describe a series of immunoprecipitation experiments which show that peptidyl boronic acids are potent inhibitors of 20S and 26S proteasomes in cultured cells. Furthermore flasks of cells were cultured in the presence of a radiolabelled peptidyl boronic acid, [^{3}H] Phenacetyl-Leu-Leu-boroLeu. Proteasome complexes were shown to be the only tight-binding cellular target using 100 nM [^{3}H] Phenacetyl-Leu-Leu-boroLeu. Previous studies

have linked potent inhibition of the LLVY activity of proteasomes to potent inhibition of protein degradation. Certain peptidyl aldehydes e.g. Cbz-Leu-Leu-norVal-al or Cbz-Leu-Leu-Leu-al were shown to potently inhibit the LLVY activity of 20S and 26S proteasomes. These peptidyl aldehydes also potently inhibited the degradation of casein, ubiquitinated lysozyme and the production of the NF-κB p50 protein by the 26S proteasome (Rock et al., 1994; Palombella et al., 1994). Other peptidyl aldehydes e.g. Ac-Leu-Leu-Nle-al and Ac-Leu-Leu-Met-al were less effective inhibitors of the 20S and 26S proteasome LLVY activity. They were also less effective at inhibiting the degradation of casein, ubiquitinated lysozyme and the production of the p50 protein. Results in Table 5.4 show that the Ki values for peptidyl boronic acid inhibition of the liver 20S proteasome LLVY activity, correlate well with IC50 values for the inhibition of β-amyloid production and NF-κB activation in cultured cells.

In the later sections of the chapter I will describe a series of experiments whose objective was to determine the peptidyl boronic acid modified subunits in 20S proteasomes. Cbz-Leu-Leu-boroLeu (pinacol ester) and [^{3}H] Phenacetyl-Leu-Leu-boroLeu were used to modify 20S proteasome subunits, that were separated by either HPLC or SDS-PAGE. Peptidyl boronic acid modification did not survive either HPLC or SDS-PAGE separation of the proteasome subunits. However, Cbz-Leu-Leu-Leu-vinyl sulphone (Z-LLL-VS) was used to block the incorporation of the radiolabelled inhibitor into the chymotrypsin-like catalytic sites of 20S proteasomes.

5.2 - Peptidyl boronic acids inhibit proteasome complexes in cultured cells

L-132 human embryonic lung cells were employed to determine whether peptidyl boronic acids could cross the plasma membrane to inhibit proteasome complexes. Subconfluent flasks

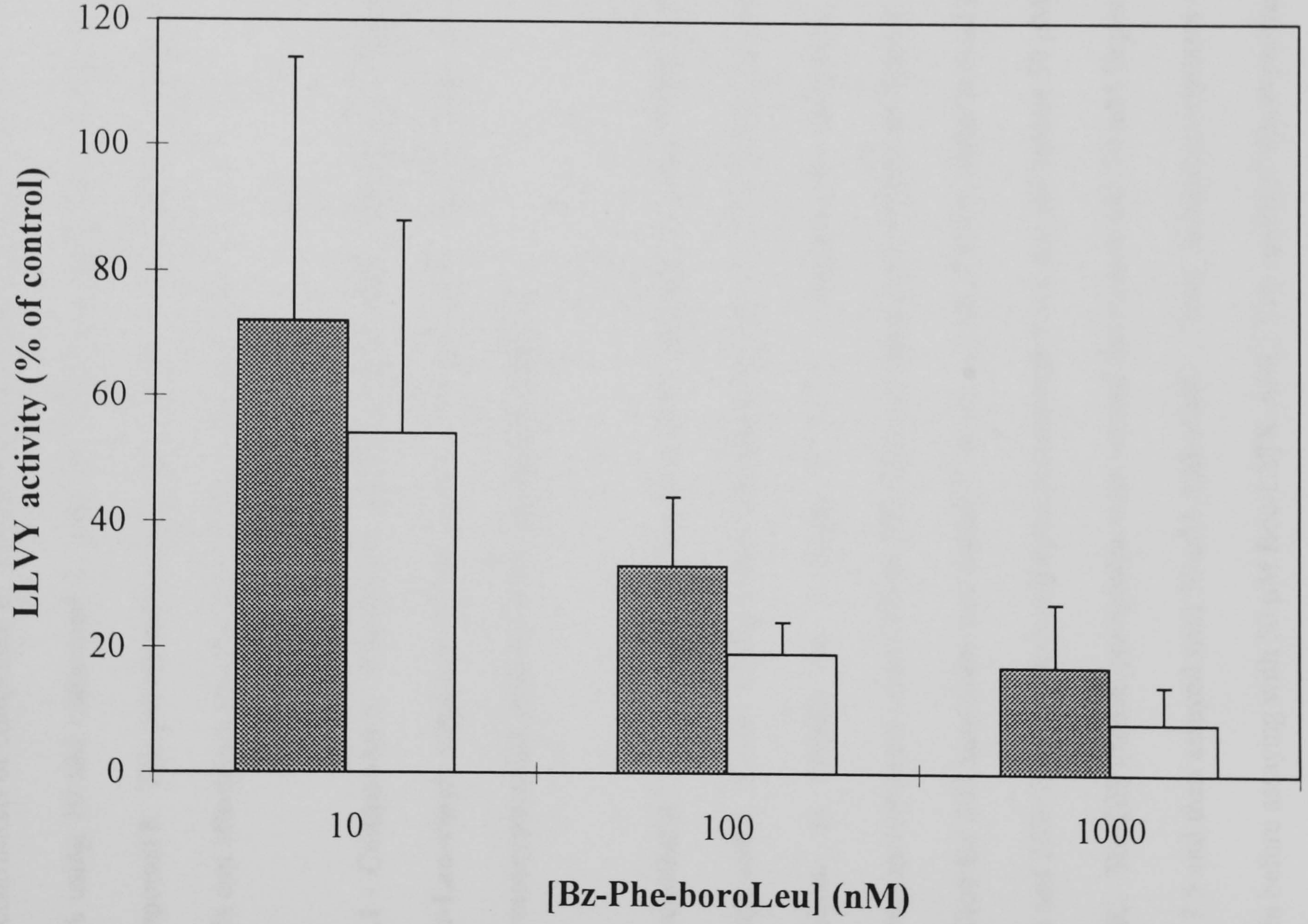
of cells were cultured in fresh medium containing DMSO (1%) or selected concentrations of peptidyl boronic acid in DMSO. Flasks were incubated for 2 hours at 37°C before washing the cells with 3 × 10 ml of PBS. The cells were lysed by two different protocols. In the first the cells were lysed using RIPA buffer (50 mM Tris/HCl, pH 8.0 containing 0.5% sodium deoxycholate, 0.1% SDS, 1% Nonidet P-40 and 150 mM NaCl). Previous studies showed that RIPA buffer causes the dissociation of 26S proteasomes into 20S proteasomes and 19S regulatory complexes (Mason et al., 1996). Alternatively cells were harvested using a trypsin/versene solution. Cells were then pelleted before lysing in 0.5 ml of 20 mM Tris buffer/HCl, pH 7.5 containing 5 mM ATP, 10% glycerol and 0.2% Nonidet P-40 (Mason et al., 1998). The contents of this buffer were chosen to maintain the structure of both the 20S and 26S proteasomes during cell lysis. Protocols for immunoprecipitation under both sets of conditions, using the MCP20 monoclonal antibody and subsequent assays are described in Chapter 2. The MCP20 antibody binds to one site on each of the 20S proteasome α-subunit rings (Kopp et al., 1993). These binding sites are exposed in the 26S proteasome, facilitating efficient immunoprecipitation of 20S and 26S proteasomes from cell lysate. In addition attachment of MCP20 to proteasomes does not affect the proteolytic activity of the complexes (Kopp et al., 1993).

Figure 5.1 shows the results of immunoprecipitation experiments using selected concentrations of Bz-Phe-boroLeu (compound 1). In vitro assay results show that the 26S proteasome LLVY activity is approximately 5-6 times greater than the 20S proteasome LLVY activity. Previous studies in this laboratory showed that a large proportion of the proteasomes immunoprecipitated from L-132 cells using the MCP20 antibody, were 26S proteasomes using the more gentle lysis conditions (Mason et al., 1998). Hence the LLVY activity of MCP20 immunoprecipitates obtained using the gentle lysis conditions, approximates to the 26S

proteasome LLVY activity. Inhibition of the 20S and 26S proteasome LLVY activities was observed with 10 and 100 nM compound 1. This confirms that compound 1 readily crosses the plasma membrane of L-132 cells and is in agreement with other cell-based experiments with this inhibitor (Christie et al., 1999). The tight association of compound 1 to the Suc-LLVY-AMC cleaving catalytic sites was previously observed in dialysis experiments (Tables 3.3 and 3.4). The fact that the association survives overnight incubation and six washing stages, shows that the off-rate is very slow. The inhibition of the 26S proteasome activity was consistently greater than the 20S proteasome activity. The reasons for this observation were unclear.

Figure 5.1 - Activity of 20S proteasomes in immunoprecipitates after treatment of L-132 cells with Bz-Phe-boroLeu (compound 1) (page 154)

Subconfluent human embryonic lung L-132 cells were cultured in fresh medium containing DMSO (1%) or appropriate amounts of compound 1 in DMSO for 2 hours at 37°C. Flasks were washed with 3 × 10 ml of PBS before conducting the immunoprecipitation of proteasome complexes under 20S or 26S conditions laid out in Chapter 2. The immunoprecipitated proteasome complexes were assayed against 50 μM Suc-LLVY-AMC. Values are from three experiments conducted in duplicate. These values are expressed as percent of control activity in samples containing no inhibitor. The results for 20S and 26S proteasomes are represented by grey and white bars respectively.


120
100
80
60
40
20
0
LLVY activity (% of control)
10
100
1000
[Bz-Phe-boroLeu] (nM)

Another peptidyl boronic acid Cbz-Leu-Leu-boroLeu (pinacol ester) (compound 8) was found to readily enter L-132 cells and inhibit proteasome complexes. Table 5.1 shows the LLVY activity of 20S proteasomes in immunoprecipitates after treatment of L-132 cells with selected concentrations of compound 8. The LLVY activity of the 20S proteasomes was inhibited by 25% using 10 nM compound 8, with the inhibition approaching completion using 1 μM compound 8. This proved that pinacol ester as well as free acid forms of the peptidyl boronic acids can effectively traverse the plasma membrane.

5.2.1 - Compound 1, Cbz-Leu-boroNle (pinacol ester) (compound 10) and Cbz-Leu-Leu-Leu-vinyl sulphone (Z-LLL-VS) can all enter L-132 cells to inhibit the chymotrypsin-like activity of proteasome complexes

Subconfluent L-132 cells were cultured in fresh medium containing DMSO (1%) or 10 μM compound 1, 10 μM Cbz-Leu-boroNle (pinacol ester) or 20 μM Cbz-Leu-Leu-Leu-vinyl sulphone in DMSO for 2 hours at 37°C. Proteasome complexes were then immunoprecipitated under 20S or 20S+26S conditions as described in Chapter 2. The basic method for 20S conditions was modified by using 5 ml of RIPA buffer instead of 1 ml. The 20S and 20S+26S immunoprecipitated proteasomes were assayed against 50 μM Suc-LLVY-AMC. The 20S immunoprecipitates were washed three times with 50 mM Hepes buffer/KOH, pH 7.5 and then assayed with 50 μM AAF-AMC. These immunoprecipitates were washed again before assaying with 50 μM Boc-LSTR-AMC. The results of this experiment are shown in Table 5.2.

Table 5.1 - Activity of 20S proteasomes in immunoprecipitates after treatment of L-132 cells with Cbz-Leu-Leu-boroLeu (pinacol ester) (compound 8)

[Cbz-Leu-Leu-boroLeu (pinacol ester)] (nM)	20S proteasome LLVY activity (% of control)
10	75
100	23
1000	4

Subconfluent human embryonic lung L-132 cells were cultured in fresh medium containing DMSO (1%) or appropriate amounts of compound 8 in DMSO for 2 hours at 37°C. Flasks were washed with 3 × 10 ml of PBS, before conducting the immunoprecipitation of proteasome complexes under 20S proteasome conditions laid out in Chapter 2. The immunoprecipitated proteasome complexes were assayed with 50 μM Suc-LLVY-AMC. Values are from a single experiment conducted in duplicate. These values are expressed as percent of control activity in samples containing no inhibitor.

Table 5.2 - Activities of 20S and 26S proteasomes immunoprecipitated from L-132 cell extract following treatment of cells with either Bz-Phe-boroLeu (compound 1), Cbz-Leu-boroNle (pinacol ester) (compound 10) or Cbz-Leu-Leu-Leu-vinyl sulphone (Z-LLL-VS)

[Inhibitor] (μM)	20S proteasome activity (% of control)			26S proteasome LLVY activity (% of control)
	LLVY	AAF	LSTR	
10 μM Bz-Phe-boroLeu	8	21	171	10
10 μM Cbz-Leu-boroNle (pinacol ester)	57	28	121	81
20 μM Cbz-Leu-Leu-Leu-vinyl sulphone	6	12	79	13

Subconfluent human embryonic lung L-132 cells were cultured in fresh medium containing DMSO (1%) or appropriate amounts of proteasome inhibitor in DMSO for 2 hours at 37°C. Flasks were washed with 3 × 10 ml of PBS before conducting the immunoprecipitation of proteasome complexes under 20S or 26S conditions laid out in Chapter 2. The basic protocol for immunoprecipitation under 20S conditions was modified by using 5 ml of RIPA buffer rather than 1 ml. The immunoprecipitated proteasome complexes were first assayed with 50 μM Suc-LLVY-AMC. The immunoprecipitates containing only 20S proteasomes were then washed three times with 50 mM Hepes buffer/KOH, pH 7.5. These immunoprecipitates were then assayed with 50 μM AAF-AMC. The 20S immunoprecipitates were washed as above before assayed with 50 μM Boc-LSTR-AMC. Values are from a single experiment conducted in duplicate. These values are expressed as percent of control activity in samples containing no inhibitor.

Inhibition of the LLVY activity of 20S and 26S proteasomes by 10 μM compound 1 was in agreement with the results in Figure 5.1. Cbz-Leu-boroNle (pinacol ester) was much less effective at inhibiting these two activities. As described in Table 3.4, the inhibition of the 20S proteasome LLVY activity by Cbz-Leu-boroNle (pinacol ester) was readily reversible during overnight dialysis. However, in this experiment the 20S proteasome LLVY activity was still inhibited by 43% after the washing stages. If the off-rate of the inhibitor was very slow in the presence of detergents during the overnight immunoprecipitation and slow in Hepes buffer solutions, then the inhibition would only be partially reversed as observed. Cbz-Leu-Leu-Leu-vinyl sulphone was previously shown to irreversibly inhibit the chymotrypsin-like, trypsin-like and PGPH activities of 20S and 26S proteasomes (Bogyo et al., 1997). Cbz-Leu-Leu-Leu-vinyl sulphone (20 μM) caused near complete inhibition of the 20S and 26S proteasome LLVY activities.

All three compounds were shown to inhibit the AAF activity of the immunoprecipitated 20S proteasomes (Table 5.2). The results for compound 1 are similar to those of a dialysis experiment shown in Table 3.4. Previous studies using peptidyl diazomethanes and peptidyl chloromethanes showed that the LLVY activity of liver 20S proteasomes was more readily inhibited than the AAF activity (Savory et al., 1993; Reidlinger et al., 1997). However these compounds were relatively weak proteasome inhibitors that were used at millimolar concentrations. The relatively high concentrations of peptidyl boronic acid or Cbz-Leu-Leu-Leu-vinyl sulphone used in this experiment, apparently caused heavy modification of the one or two types of catalytic site that catalyse the AAF activity. This modification was only slowly reversed during the procedure. Both compound 1 and Cbz-Leu-boroNle (pinacol ester) caused stimulation of the 20S proteasome LSTR activity. These results are in good agreement with the corresponding results in Table 3.4. Stimulation of the LSTR activity may result from

inhibitor binding to non-trypsin-like catalytic sites, causing conformational changes that stimulate the trypsin-like activity. Cbz-Leu-Leu-Leu-vinyl sulphone caused moderate inhibition of the LSTR activity. This inhibitor was previously shown to inhibit the trypsin-like activity of 20S proteasomes measured with the Boc-LRR-AMC substrate (Bogyo et al.,1997).

5.3 - Peptidyl boronic acids readily enter IMR-32 and IMR-32 APP-751 cells to inhibit proteasome complexes

As part of my CASE studentship with SmithKline Beecham, I was given the opportunity to work at the main SmithKline Beecham site in Harlow, Essex. During my visit to Harlow I performed the immunoprecipitation experiments with two neural cell lines and 2-pyrazinylcarbonyl-Phe-boroLeu. The first cell line was the human neuroblastoma cell line IMR-32. A murine tetraploid neuronal cell line named IMR-32 APP-751 was also used. Table 5.3 shows a single set of results for the IMR-32 cells. 2-pyrazinylcarbonyl-Phe-boroLeu was added to the culture medium for 24 rather than 2 hours in these experiments. Immunoprecipitated 20S or 26S proteasomes exhibited an IC50 value of approx. 5 nM for inhibition of the LLVY activity. The IC50 value for L-132 cells using compound 1 and a 2 hour incubation period was approx. 40 nM. The same experimental protocol was initially tried with the IMR-32 APP-751 cells. However, the MCP20 antibody which was raised in mice, does not immunoprecipitate mouse proteasome complexes. A polyclonal anti-proteasome antibody, Ab186 that was raised in rabbit was therefore used for immunoprecipitation. Figure 5.2 illustrates the results of two experiments in which IMR-32 APP-751 cells were incubated with 2-pyrazinylcarbonyl-Phe-boroLeu for 24 hours, before immunoprecipitating proteasome complexes with Ab186. Immunoprecipitated 20S or 26S proteasomes exhibited an IC50 value of approx. 20 nM for inhibition of the LLVY activity. This is in good agreement with

the results for the IMR-32 cells. Therefore proteasome complexes can be potently and reproducibly inhibited by peptidyl boronic acids, in three separate cell lines from two different species.

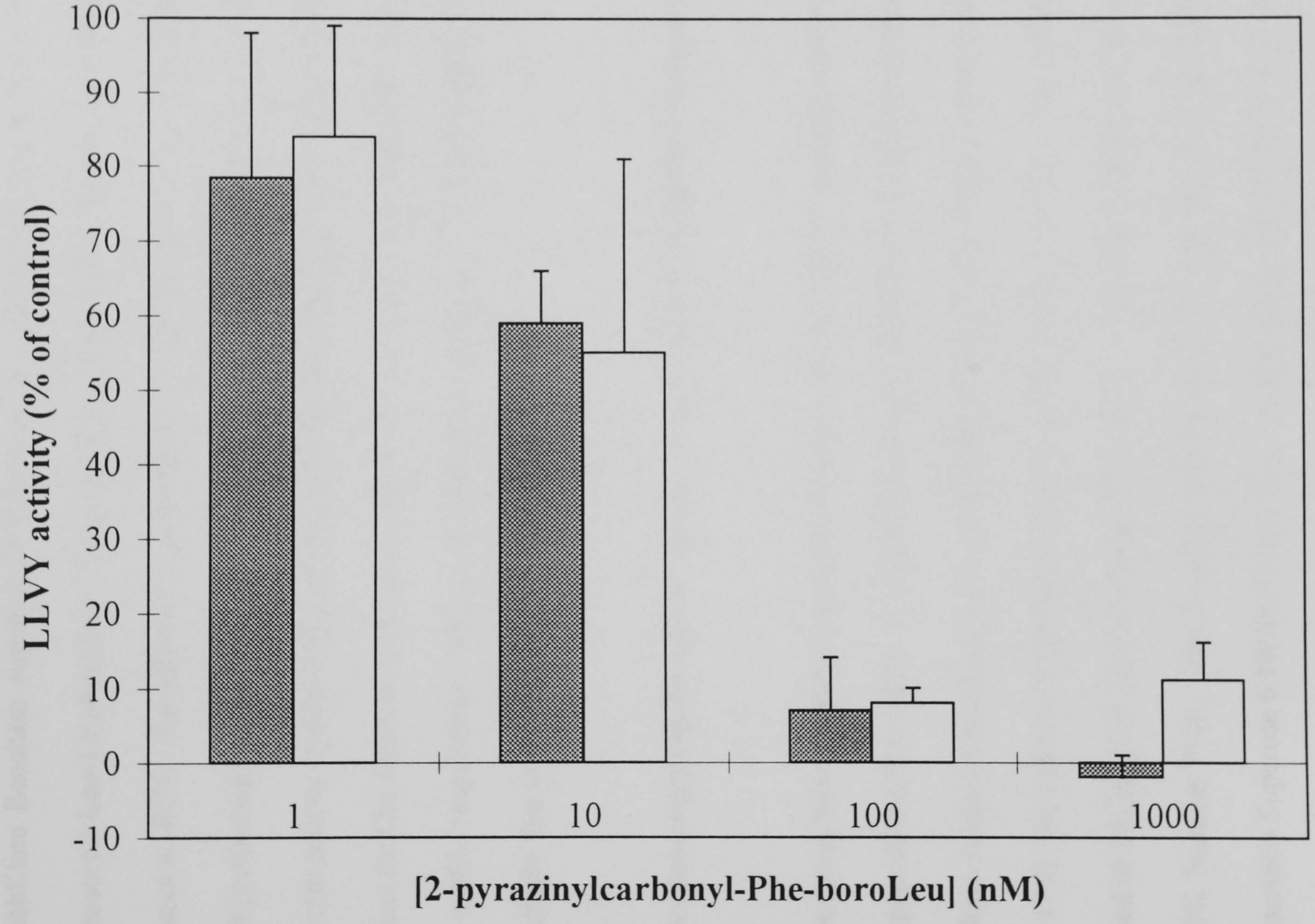
Table 5.3 - Activities of proteasomes immunoprecipitated from IMR-32 cell extract following treatment of cells with 2-pyrazinylcarbonyl-Phe-boroLeu

[2-pyrazinylcarbonyl-Phe-boroLeu] (nM)	20S proteasome LLVY activity (% of control)	26S proteasome LLVY activity (% of control)
1	115	69
10	38	19
100	24	14
1000	15	N.D.

Subconfluent human neuroblastoma IMR-32 cells were cultured in fresh medium containing DMSO (1%) or appropriate amounts of 2-pyrazinylcarbonyl-Phe-boroLeu in DMSO for 24 hours at 37°C. Flasks were washed with 3 × 10 ml of PBS before conducting the immunoprecipitation of proteasome complexes under 20S or 26S conditions, laid out in Chapter 2. The immunoprecipitated proteasome complexes were assayed with 50 μM Suc-LLVY-AMC. Values are from a single experiment conducted in duplicate. These values are expressed as percent of control activity in samples containing no inhibitor. N.D. - not determined.

Figure 5.2 - Activity of 20S proteasomes in immunoprecipitates after treatment of IMR-32 APP-751 cells with 2-pyrazinylcarbonyl-Phe-boroLeu (page 163)

Subconfluent murine tetraploid neuronal IMR-32 APP-751 cells were cultured in fresh medium containing DMSO (1%) or appropriate amounts of 2-pyrazinylcarbonyl-Phe-boroLeu in DMSO for 24 hours at 37°C. Flasks were washed with 3 × 10 ml of PBS before conducting the immunoprecipitation of proteasome complexes under 20S or 26S conditions, laid out in Chapter 2. The immunoprecipitated proteasome complexes were assayed against 50 μM Suc-LLVY-AMC. Values are from two experiments conducted in duplicate. These values are expressed as percent of control activity in samples containing no inhibitor. The results for 20S and 26S proteasomes are represented by grey and white bars respectively. The error bars represent the range of readings over the two experiments.


100
90
80
70
60
50
40
30
20
10
0
-10
LLVY activity (% of control)
1
10
100
1000
[2-pyrazinylcarbonyl-Phe-boroLeu] (nM)

5.4 - Effect of [^{3}H] Phenacetyl-Leu-Leu-boroLeu (radiolabelled inhibitor) on the LLVY activity of liver 20S proteasomes

SmithKline Beecham supplied 18.5 MBq of a radiolabelled peptidyl boronic acid [^{3}H] Phenacetyl-Leu-Leu-boroLeu. The peptidyl boronic acid was stored as a 1 mM solution in ethanol at -80°C. The effect of 1 μM radiolabelled inhibitor on the LLVY activity of 1 μg liver 20S proteasome was determined. These assays were conducted as described in Chapter 2. The percent activity relative to the control for three experiments was 37 ± 5%. The corresponding values for Cbz-Leu-Leu-boroLeu and Cbz-Leu-Leu-boroLeu (pinacol ester) were 25 ± 2% and 22 ± 8% respectively. The small decrease in potency of the radiolabelled inhibitor was probably due to the different N-terminal group.

5.5 - Investigating the cellular targets of a radiolabelled peptidyl boronic acid

The radiolabelled inhibitor was used to confirm that proteasome complexes are a cellular target of peptidyl boronic acids. A single flask of subconfluent L-132 cells was cultured with fresh culture medium containing 1 μM radiolabelled inhibitor. Following a 2 hour incubation period at 37°C, the cells were harvested using a trypsin/versene solution. The pelleted cells were lysed in 200 μl of 20 mM Tris buffer/HCl, pH 7.5 containing 5 mM ATP, 10% glycerol and 0.2% Nonidet P-40. The resultant cell extract was fractionated by gel filtration using a Pharmacia Superose 6 10/30 column. The column was equilibrated in 20 mM Tris buffer/HCl, pH 7.5 containing 5 mM ATP, 10% glycerol and 150 mM KCl. ATP and glycerol were used in the lysis and fractionation buffers to help maintain the structure of the 26S proteasome (Figure 5.3 on page 168). In previous experiments the structure of the PA28-20S proteasome

complex was shown not to survive gel filtration using the listed lysis and fractionation buffers (Paul Brooks, personal communication). The 26S proteasome was shown using Western blotting to elute after approximately 10-12 ml (fractions 10-12) from the start of fractionation, using the Superose 6 column (Brooks et al., in press). The smaller 20S proteasome elutes after approximately 12-14 ml (fractions 12-14). There are peaks of LLVY activity associated with these fractions, and the LLVY substrate is also hydrolysed by an enzyme eluting in fractions 16-18. The majority of the radiolabelled inhibitor was associated with the 20S proteasome in fractions 12-14, but fraction 17 also contained radiolabelled material.

The minor peak of radioactivity observed in fraction 17, coincided with a peak of LLVY activity that was observed only in the absence of 0.02% SDS. The radioactivity in fraction 17 was approx. 5% of the total recorded activity. Figure 5.4 (page 169) shows the results of a similar fractionation experiment using 100 nM radiolabelled inhibitor. At the lower inhibitor concentration the minor peak of radioactivity is eliminated and all the bound inhibitor was associated with 20S proteasomes. Figure 5.5 (page 170) shows the results of a control fractionation experiment, where the flask of cells was cultured with fresh medium containing no radiolabelled inhibitor for 2 hours. When comparing Figures 5.4 and 5.5 it is clear that the 26S proteasomes in fractions 10 and 11 are not inhibited by the radiolabelled inhibitor. This correlates with the very low level of radioactivity in fractions 10 and 11 of Figure 5.4. Greater levels of radioactivity were measured in fractions 13 and 14 of Figure 5.4 compared to fractions 10-12. When comparing Figures 5.4 and 5.5 this modification did give rise to a significant decrease in the LLVY activity of 20S proteasomes in fraction 13 and 14. For fraction 13 the percent decrease in activity was calculated in two stages: $0.88/1.46 \times 100\% = 60\%$ and then $100\%-60\% = 40\%$. For fraction 14: $0.72/1.03 \times 100\% = 70\%$ and then $100\%-70\% = 30\%$. It should also be noted that a significant amount of radiolabelled inhibitor in

Figure 5.4, has run through in the column volume. This is presumably radiolabelled inhibitor that has dissociated from 20S and particularly 26S proteasomes during the column run. A small amount of radiolabelled inhibitor has run through in the column volume of the experiment illustrated in Figure 5.3. In vitro assay results show that 1 μM radiolabelled inhibitor does not inhibit the trypsin-like and PGPH activities of liver 20S proteasomes (Table 5.7). The chymotrypsin-like activity measured with the Suc-LLVY-AMC or AAF-AMC substrates was substantially inhibited. Moreover, this inhibition was not readily reversed during rapid gel filtration. If the rate of radiolabelled inhibitor detachment from 26S proteasomes was faster than 20S proteasomes, the 20S proteasomes would apparently be modified to a greater extent. It should be noted that this type of experiment may not detect other cellular targets, if the binding of radiolabelled inhibitor is readily reversed.

Figure 5.3 - Fractionation of cell extract following treatment of L-132 cells with 1 μM [^{3}H] Phenacetyl-Leu-Leu-boroLeu (radiolabelled inhibitor) (page 168)

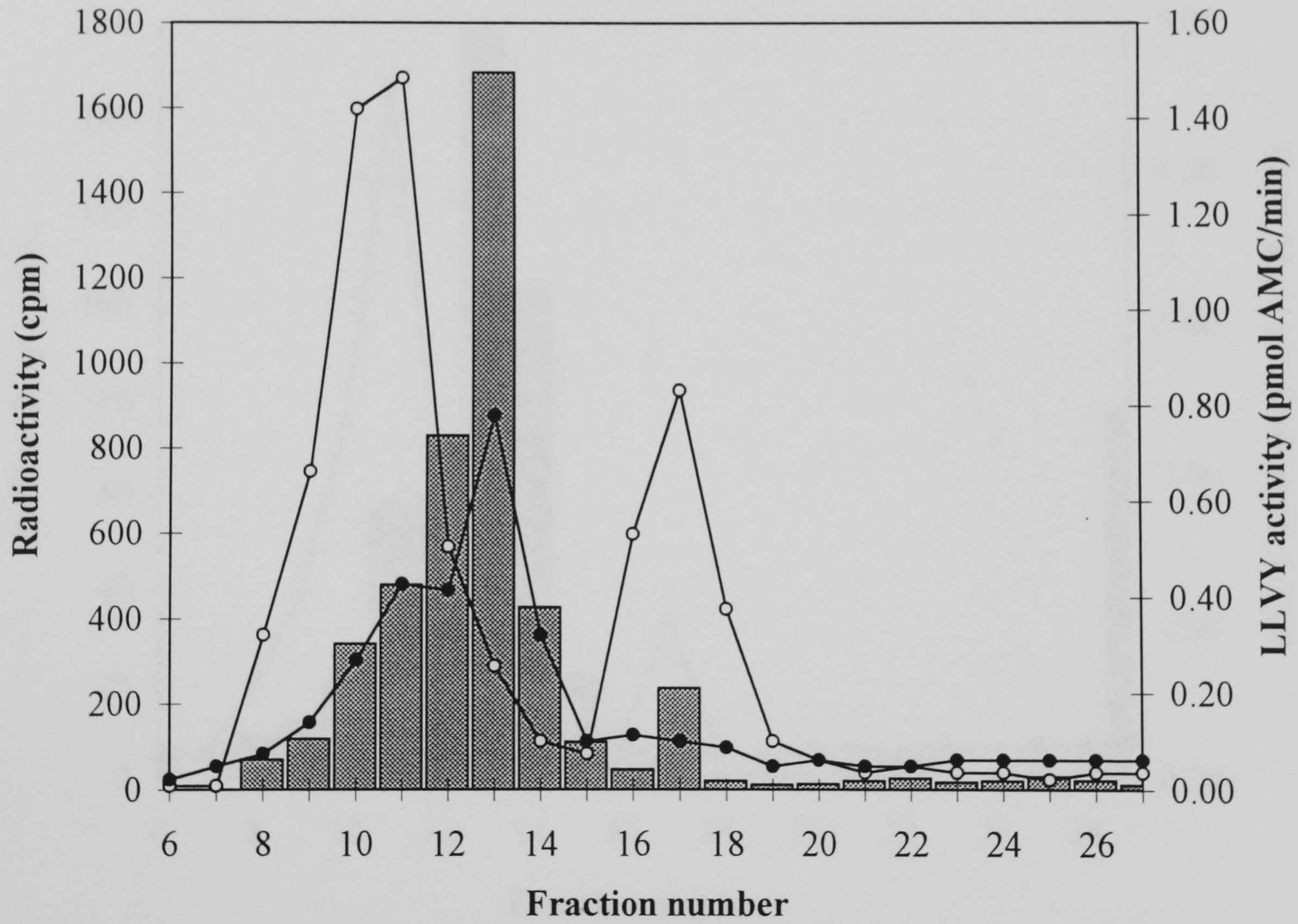
One flask of subconfluent human embryonic lung L-132 cells was cultured in fresh medium containing 1 μM radiolabelled inhibitor for 2 hours at 37°C. The cells were harvested using a trypsin/versene solution and then pelleted. The pelleted cells were lysed in 200 μl of 20 mM Tris buffer/HCl, pH 7.5 containing 5 mM ATP, 10% glycerol and 0.2% Nonidet P-40. The cell extract was fractionated by gel filtration using a Pharmacia Superose 6 10/30 column. The column was equilibrated with 50 ml of 20 mM Tris buffer/HCl, pH 7.5 containing 5 mM ATP, 10% glycerol and 150 mM KCl. Buffer was pumped through the column at a rate of 0.2 ml/min and 1 ml fractions were collected. Aliquots (350 μl) of each fraction was mixed with 12 ml of scintillation cocktail before conducting radioactive counting (filled bars) as described in Chapter 2. Two, 150 μl aliquots of each fraction were assayed with 40 μM Suc-LLVY-AMC, either in the absence (open circles) or presence (filled circles) of 0.02% SDS. The assays were incubated at 37°C for 30 minutes before stopping and reading as described in Chapter 2.

Figure 5.4 - Fractionation of cell extract following treatment of L-132 cells with 100 nM [^{3}H] Phenacetyl-Leu-Leu-boroLeu (radiolabelled inhibitor) (page 169)

An experiment was performed as described in the Legend to Figure 5.3 with the L-132 cells being cultured in fresh medium containing 100 nM radiolabelled inhibitor for 2 hours at 37°C.

Figure 5.5 - Fractionation of L-132 cell extract on a Superose 6 gel filtration column (page 170)

An experiment was performed as described in the Legend to Figure 5.3 with the L-132 cells being cultured in fresh medium containing 5 μl ethanol for 2 hours at 37°C. The fractions were only assayed for peptidase activities in this experiment.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1400
1200
1000
800
600
400
200
0
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1.20
1.00
0.80
0.60
0.40
0.20
0.00
LLVY activity (pmol AMC/min)
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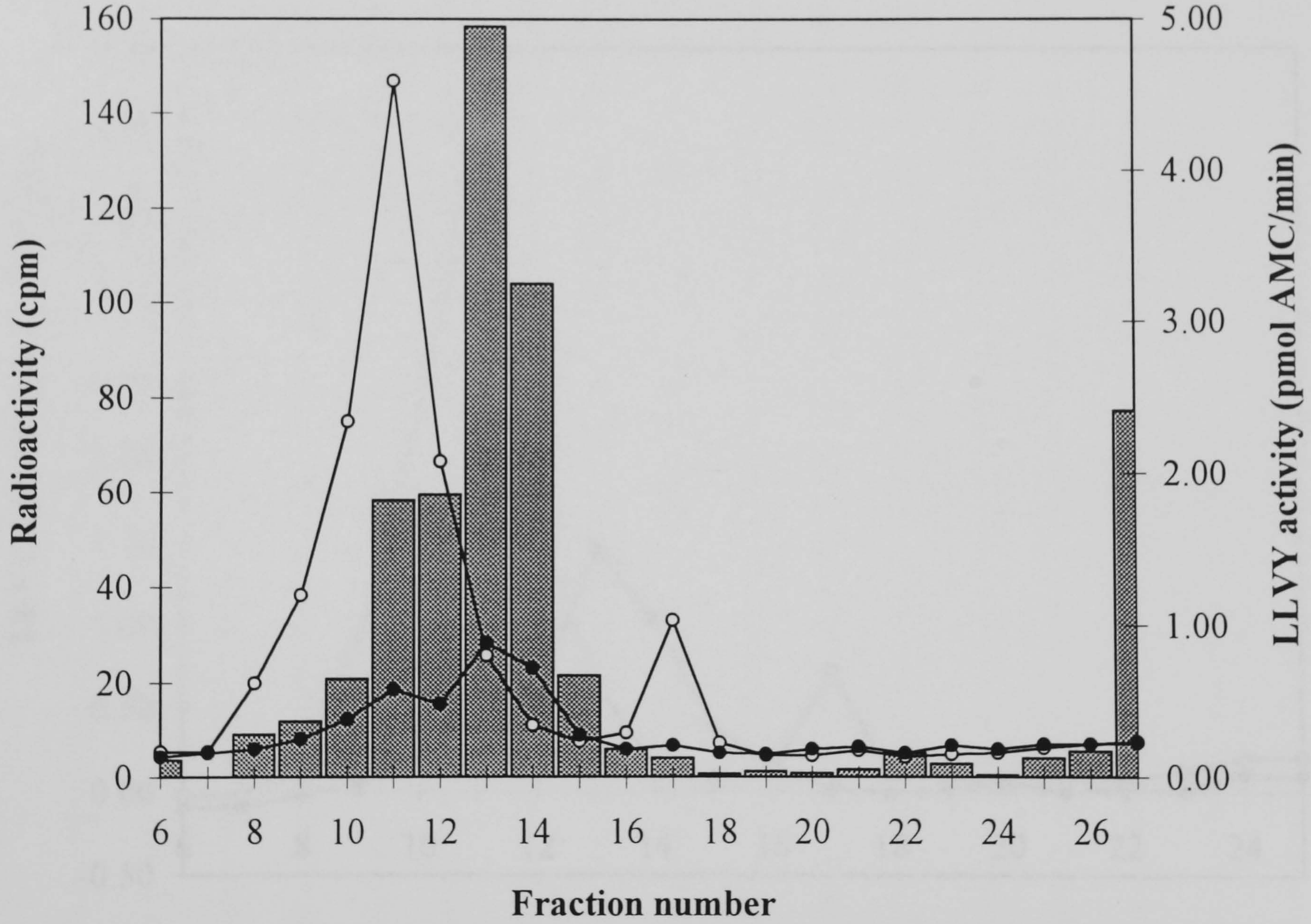
8
10
12
14
16
18
20
22
24
26
Fraction number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100
80
60
40
20
0
Radioactivity (cpm)
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4.00
3.00
2.00
1.00
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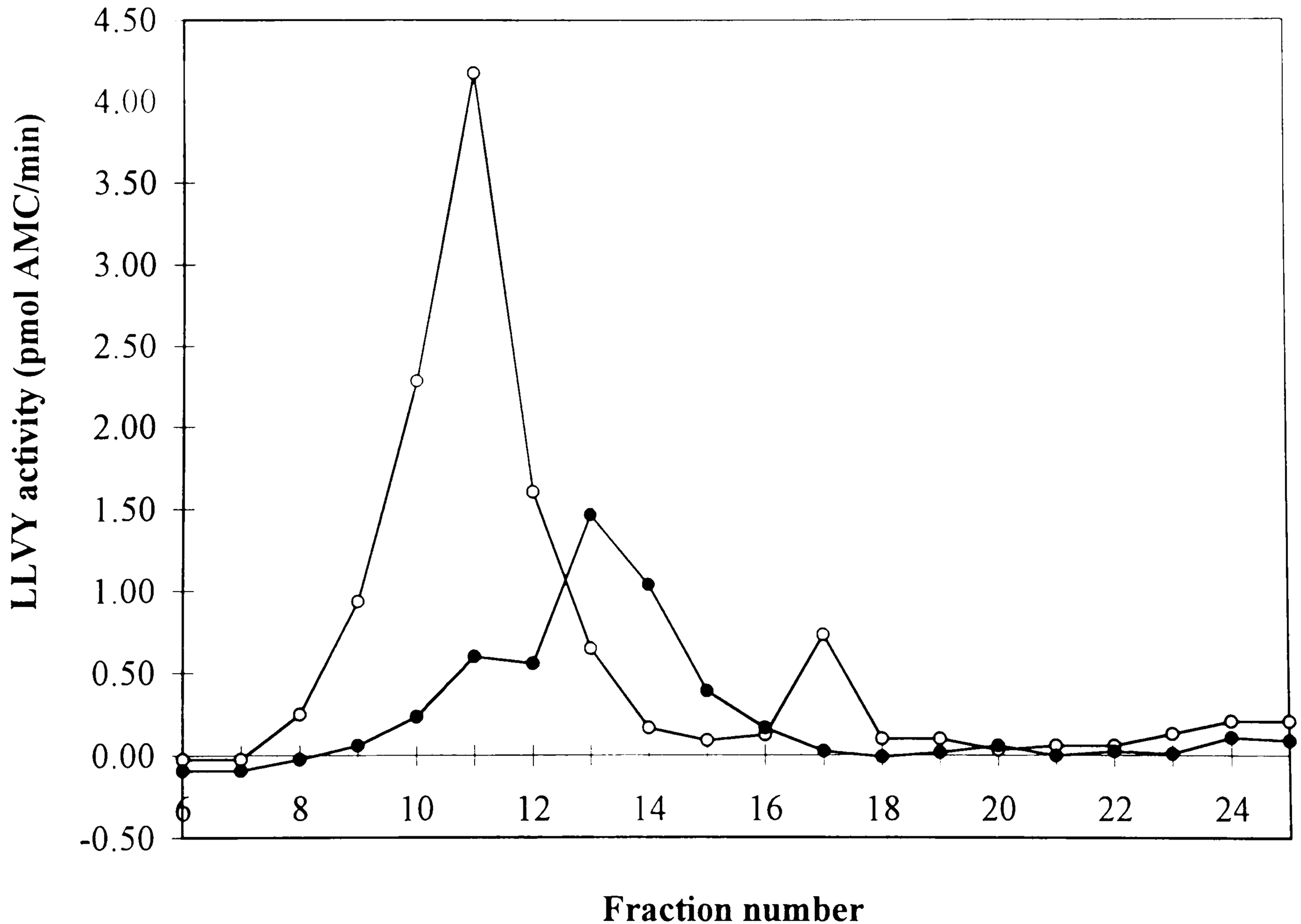
LLVY activity (pmol AMC/min)
6
8
10
12
14
16
18
20
22
24
26
Fraction number


LLVY activity (pmol AMC/min)
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4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
-0.50
6
8
10
12
14
16
18
20
22
24
Fraction number

5.6 - Kinetic constants for inhibition of purified 20S proteasomes by peptidyl boronic acids are comparable to IC50 values for inhibition of proteolytic reactions in cultured cells

Table 5.4 compares Ki values for inhibition of the liver 20S proteasome LLVY activity with IC50 values for inhibition of β-amyloid production or NF-κB activation in cultured cells. The Ki values were similar in magnitude to the IC50 values for all the listed peptidyl boronic acids except Cbz-Leu-boroPhe (pinacol ester) (NF-κB assay only) and Cbz-nitroArg-boroLeu (pinacol ester). The presence of the non-physiological nitro-Arg residue presumably blocked the entry of Cbz-nitroArg-boroLeu (pinacol ester) into cultured cells. The three relatively poor inhibitors of the LLVY activity listed in panel B were relatively poor inhibitors of the other proteolytic reactions. The amyloid precursor protein (APP) is usually cleaved in neural cells by an α-secretase, to yield a large soluble N-terminal fragment named sAPPα and a membrane-bound polypeptide. Alternatively β- and γ-secretases cleave APP to yield β-amyloid and a soluble APP polypeptide (Haass & Selkoe, 1998). Several forms of β-amyloid are produced with Aβ40 and Aβ42 being the predominant species (Christie et al., 1999). Aβ42 and Aβ43 are major components of senile plaques in cases of Alzheimer's disease and Down's syndrome (Hamazaki et al., 1998). Peptidyl boronic acids are proposed to inhibit the γ-secretase activity through inhibiting proteasome complexes, because they inhibit the production of Aβ and the p3 peptide that is produced by the action of α- and γ-secretases (Christie et al., 1999). They have little effect on the production of sAPPα or the soluble APP protein produced by the β-secretase.

Table 5.4 - Kinetic constants for inhibition of purified 20S proteasomes by peptidyl boronic acids are comparable to IC50 values for inhibition of proteolytic reactions in whole cells

Panel A

Peptidyl boronic acid (compound no.)	Ki for inhibition of the liver 20S proteasome LLVY activity (nM)	IC50 for inhibition of NF-κB activation (nM)	IC50 for inhibition of β-amyloid production (nM)
Bz-Phe-boroLeu (compound 1)	17 ± 4	74, 96	6, 14
Bz-Phe-boroLeu (pinane diol ester) (compound 2)	53 ± 5	92	19
Cbz-nitroArg-boroLeu (pinacol ester) (compound 12)	39 ± 4	>10,000	>3,000
Bz-Val(Ph)-boroLeu (pinane diol ester) (compound 14)	201 ± 87	670	48
Cbz-Leu-boroPhe (pinacol ester) (compound 9)	84 ± 12	2,300	295
Cbz-Leu-Leu-boroLeu (compound 7)	46 ± 8	310	47, 174

Cbz-Leu-Leu-boroLeu (pinacol ester) (compound 8)	99 ± 19	140, 200	34, 86

Panel B

Peptidyl boronic acid (compound no.)	LLVY activity (% of control) at 1 μM compound	IC50 for inhibition of NF-κB activation (nM)	IC50 for inhibition of β-amyloid production (nM)
$PhSO_2$-Leu-boroLeu (pinacol ester) (compound 15)	54	6,400	>1,000
Cbz-Leu-t-butylboroLeu (pinacol ester) (compound 16)	73	>10,000	N.D.
Cbz-Pro-boroLeu (pinacol ester) (compound 17)	91	>10,000	>10,000

The Ki and LLVY activity values were determined as described in the legends to Tables 4.1 and 4.2. The Ki values are given as the mean ± S.D. from three or four separate experiments. The LLVY activity data is given as the average of two separate experiments. IC50 values for inhibiting the activation of NF-κB or the production of β-amyloid were determined by Scientists at SmithKline Beecham as described on page 174 or in (Christie et al., 1999). These results are from one or two experiments and are either published in (Christie et al., 1999), (Gardner et al., 2000) or supplied by Dr G. Christie of SmithKline Beecham. N.D. - not determined.

Assay of NF-κB activation

A luciferase reporter plasmid containing the IL-8 "core" promoter was engineered and stably transfected in U937 cells as previously described (Breton & Chabot-Fletcher, 1997). Transcription of the reporter construct from the IL-8 "core" promoter is stimulated by active p50-p65 NF-κB complexes. These complexes are produced through the proteasome dependent degradation of inhibitory IκB proteins and proteasome dependent production of mature NF-κB p50 proteins (See sections 1.1 and 1.9). Transfected U937 cells were twice centrifuged at 300 ×g for 5 minutes and resuspended in RPMI 1640 medium with 10% foetal bovine serum to a density of 1×10^6 cells/ml. Aliquots (200 µl) were added to the wells of an opaque 96-well filter bottom plate. Inhibitor or dimethyl sulphoxide carrier (1 µl) was added to the appropriate wells in triplicate and the plate was incubated at 37°C, 5% CO_2 for 30 minutes. The stimulus was added (5 ng/ml tumor necrosis factor-α) and the samples were incubated for 5 hours at 37°C, 5% CO_2. At the end of the incubation, the medium was removed by filtration and the cells were washed twice with PBS without Ca^{2+}, Mg^{2+}. The resulting cell pellets were lysed in 20 µl of 1 × lysis buffer (Promega Corporation, Southampton, U.K.) and incubated for 15 minutes at room temperature. Luciferase activity was measured in a MicroLumat LB 96P luminometer (EG&G Berthold, Bad Wilbad, Germany) which dispensed 100 µl of luciferase assay reagent (Promega Corporation, Southampton, U.K.) into each well and recorded the integrated light output for 20 seconds. Light output was measured in relative light units.

5.7 - HPLC separation of Cbz-Leu-Leu-boroLeu (pinacol ester) (compound 8) labelled liver 20S proteasomes

Peptidyl vinyl sulphones are irreversible inhibitors of the chymotryptic, tryptic and PGPH activities of proteasome complexes (Bogyo et al., 1997; Bogyo et al., 1998). McCormack and coworkers (1997) showed that Cbz-Leu-Leu-Leu-vinyl sulphone (Z-LLL-VS) inhibited the chymotrypsin-like activity of recombinant Rhodococcus 20S proteasomes. These proteasomes were composed of the Rhodococcus α_2 and β_2 subunits in the standard $\alpha_7\beta_7\beta_7\alpha_7$ arrangement. Rhodococcus 20S proteasomes were incubated in the absence or presence of 50 μM Z-LLL-VS, before separating the proteasome subunits on a D4 reverse phase HPLC column. Z-LLL-VS modification caused a 1.9 minute shift in the elution position of the β subunit, without effecting the elution position of the α subunit (McCormack et al., 1997). The proteasome inhibitor lactacystin did not cause a similar shift. Preincubation of Rhodococcus 20S proteasomes with lactacystin followed by Z-LLL-VS, prevented the shift in elution position of the β-subunit.

It was of interest to determine whether binding of a tripeptidyl boronic acid to one or more catalytic β subunits would also shift the elution position(s) on a HPLC trace. Cbz-Leu-Leu-boroLeu (pinacol ester) (compound 8) had the same tri-Leu peptide arrangement as Z-LLL-VS and was used in these experiments. In the first experiment 280 μg of liver 20S proteasome was dialysed against 50 mM Hepes buffer/KOH, pH 7.5. Dialysed 20S proteasome (270 μg) was then incubated with 10 μM compound 8. Compound 8 (10 μM) was used to inhibit the high (1.6 μM) concentration of 20S proteasome in the incubation mixture. After incubation at 25°C for 30 minutes, 2 μg portions of compound 8 treated and untreated 20S proteasome

were assayed against 40 μM Suc-LLVY-AMC as described in Chapter 2. The recorded percent inhibition was 73%. This result confirmed that compound 8 caused inhibition of the LLVY activity even at the high 20S proteasome concentration. Compound 8 treated 20S proteasome (200 μg) was separated on a Vydac C4 reverse-phase HPLC column as described in Chapter 2. The Absorbance (220 nm) versus elution time plot for this column run is shown in Figure 5.6 (page 178). In the first control run, 280 μg of liver 20S proteasome was dialysed against 50 mM Hepes buffer/KOH, pH 7.5. Dialysed 20S proteasome (200 μg) was prepared and then separated on the Vydac C4 column. The corresponding plot for this column run is shown in Figure 5.7 (page 179). Comparison of Figures 5.6 and 5.7 revealed the fact that the peak at 47.5 minutes in the control run, had disappeared in the compound 8 run and a new early peak at 53 minutes was visible in this profile. Previous separations of liver 20S proteasomes on a Vydac C4 column were analysed by immunoblotting. The first protein peak was identified as LMP7 (Reidlinger et al., 1997). Experiments utilising unlabelled and radiolabelled peptidyl vinyl sulphones, clearly suggested that the catalytic β-subunits LMP7 and X/MB1 catalyse the chymotrypsin-like activity of mammalian 20S proteasomes (Table 1.3) (Bogyo et al., 1998).

However, the two column runs were repeated and the Absorbance (220 nm) versus elution time plots for these HPLC separations are shown in Figures 5.8 and 5.9 (pages 180 and 181). The position of the putative LMP7 peak in the compound 8 and control runs was 50 and 51 minutes respectively. Therefore it was concluded that this peak exhibited a degree of variation between 47 and 53 minutes. These results did not disprove the theory that compound 8 remained bound to the catalytic β subunits, during HPLC separation. Hence the radiolabelled inhibitor was used in the next set of experiments.

Figure 5.6 - Separation of Cbz-Leu-Leu-boroLeu (pinacol ester) (compound 8) modified 20S proteasome subunits by reverse-phase HPLC (page 178)

Liver 20S proteasome (280 μg; 1.6 μM) was dialysed against 50 mM Hepes buffer/KOH, pH 7.5 using a 75 kDa collodion bag. Dialysis was conducted at 4°C for 3 hours. Dialysed proteasome (270 μg) was then incubated with 10 μM compound 8 for 30 minutes at 25°C. Aliquots (2 μg) of untreated and compound 8 treated 20S proteasome were then assayed against 40 μM Suc-LLVY-AMC. The assays were conducted in duplicate as described in Chapter 2. Compound 8 modification caused 73% inhibition of the LLVY activity. The compound 8 treated 20S proteasome sample was then prepared for injection onto a Vydac C4 reverse-phase HPLC column. Details of the preparation and subsequent separation of 200 μg 20S proteasome are given in Chapter 2. The figure illustrates the Absorbance (220 nm) against elution time (minutes) plot. Absorbance peaks are marked with corresponding retention times in minutes.

Figure 5.7 - Separation of 20S proteasome subunits by reverse-phase HPLC (page 179)

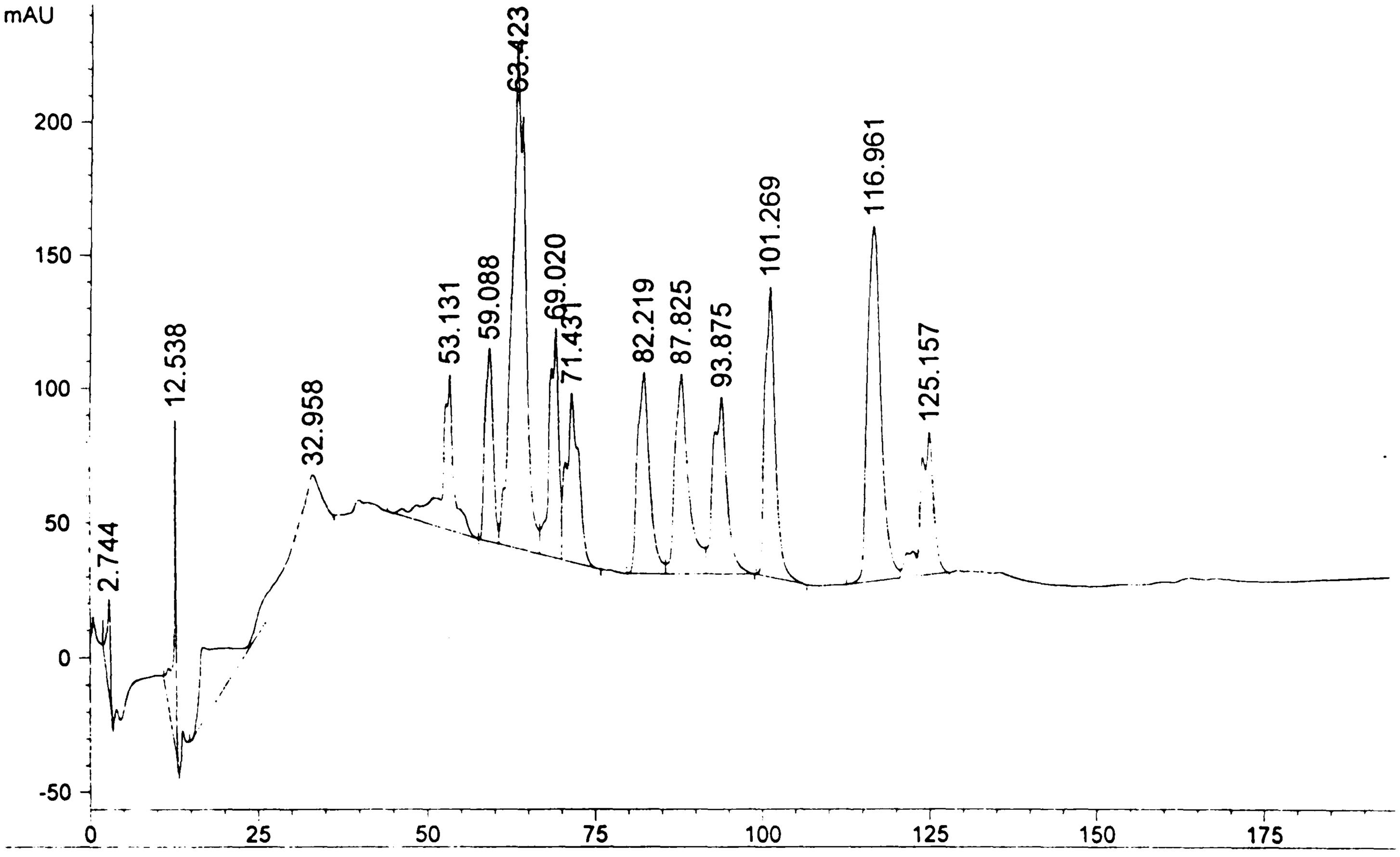
Liver 20S proteasome (280 μg; 1.6 μM) was dialysed against 50 mM Hepes buffer/KOH, pH 7.5 using a 75 kDa collodion bag. Dialysis was conducted at 4°C for 3 hours. The dialysed 20S proteasome sample was then prepared for injection onto a Vydac C4 reverse-phase HPLC column. Details of the preparation and subsequent separation of 200 μg 20S proteasome are given in Chapter 2. The figure illustrates the Absorbance (220 nm) against elution time (minutes) plot. Absorbance peaks are marked with corresponding retention times in minutes.

Figure 5.8 - Separation of Cbz-Leu-Leu-boroLeu (pinacol ester) (compound 8) modified 20S proteasome subunits by reverse-phase HPLC (page 180)

An experiment was performed as described in the legend to Figure 5.6. Compound 8 modification caused 82% inhibition of the LLVY activity.

Figure 5.9 - Separation of 20S proteasome subunits by reverse-phase HPLC (page 181)

An experiment was performed as described in the legend to Figure 5.7.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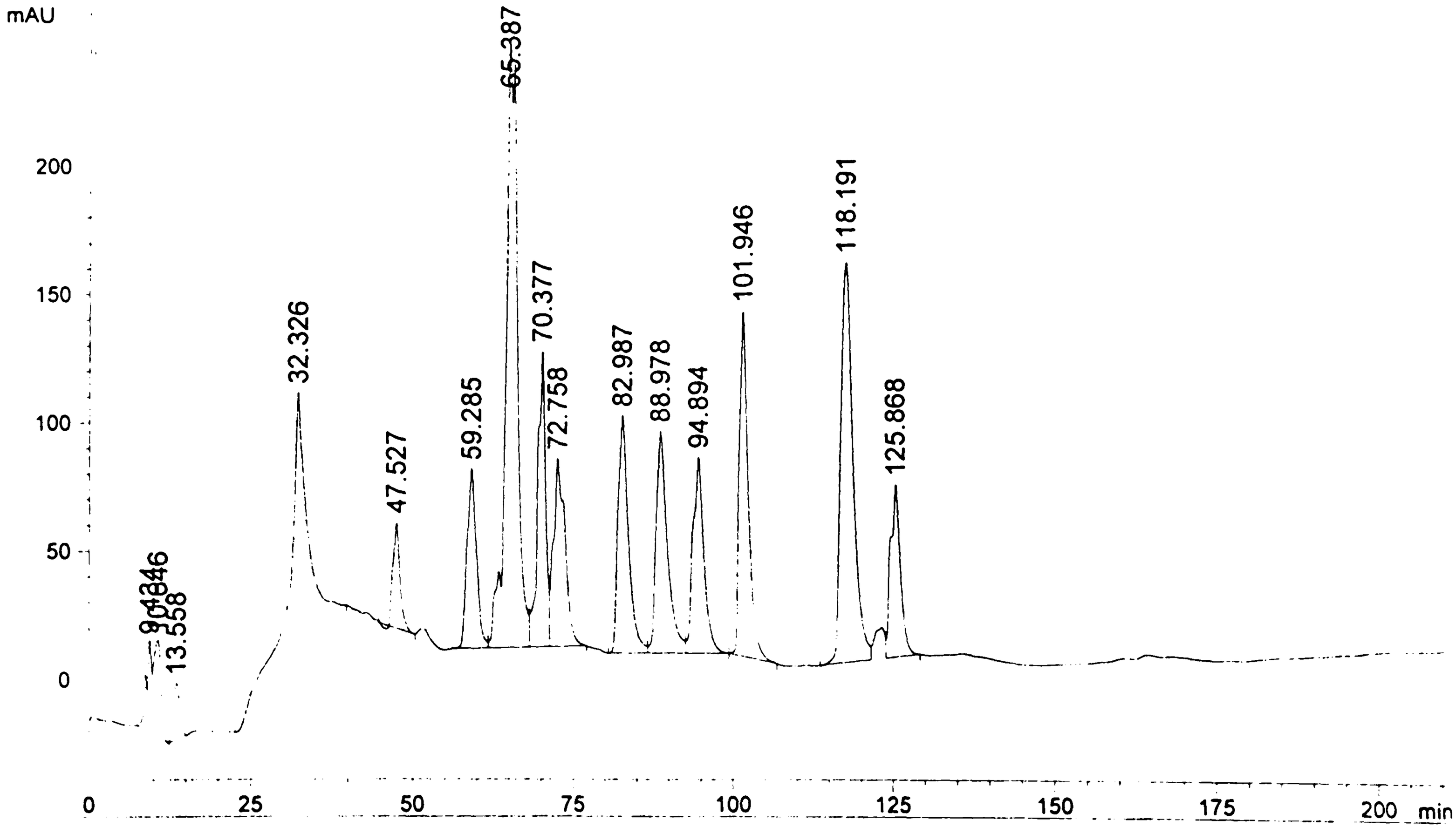
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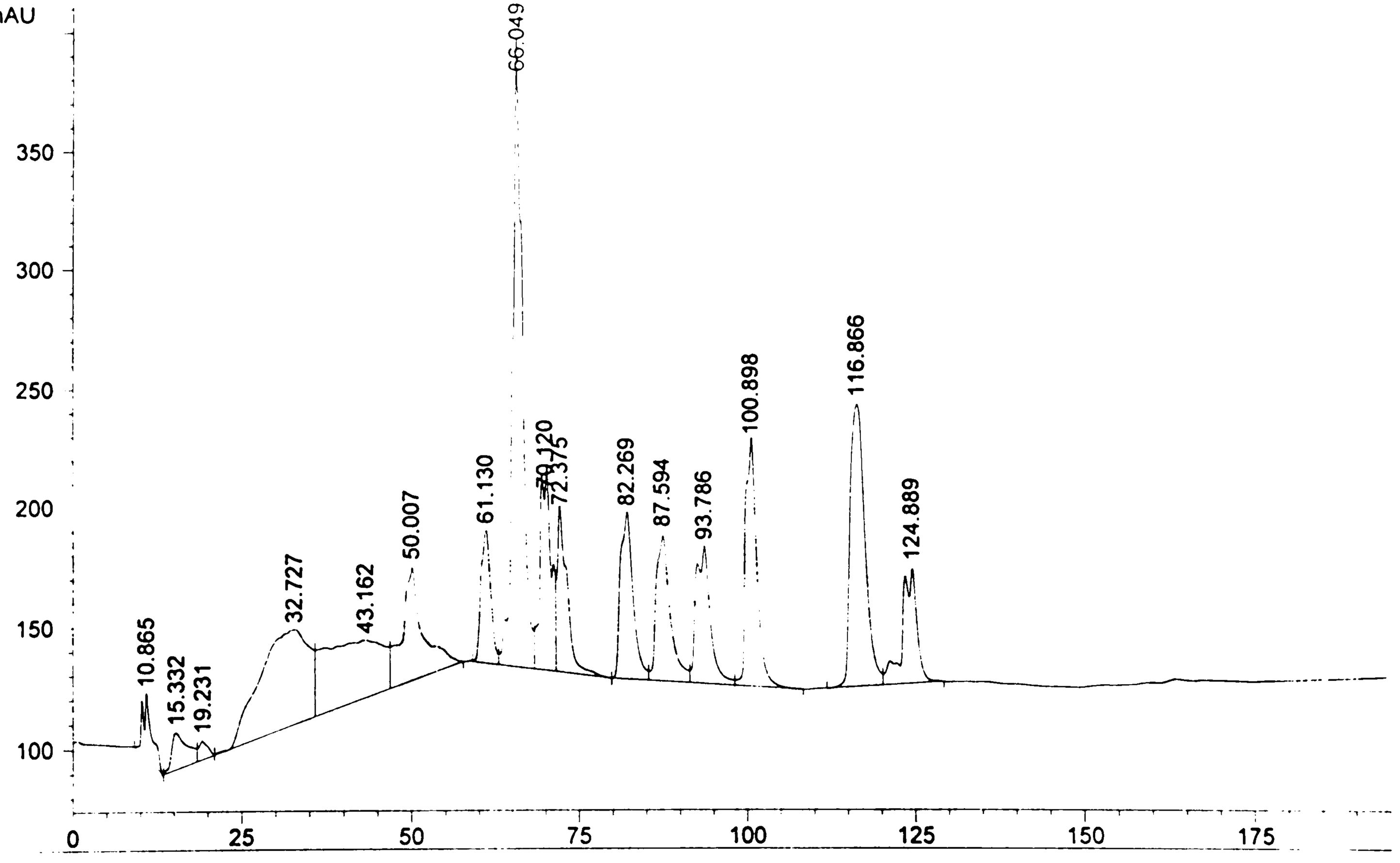
100
125
150
175
200
min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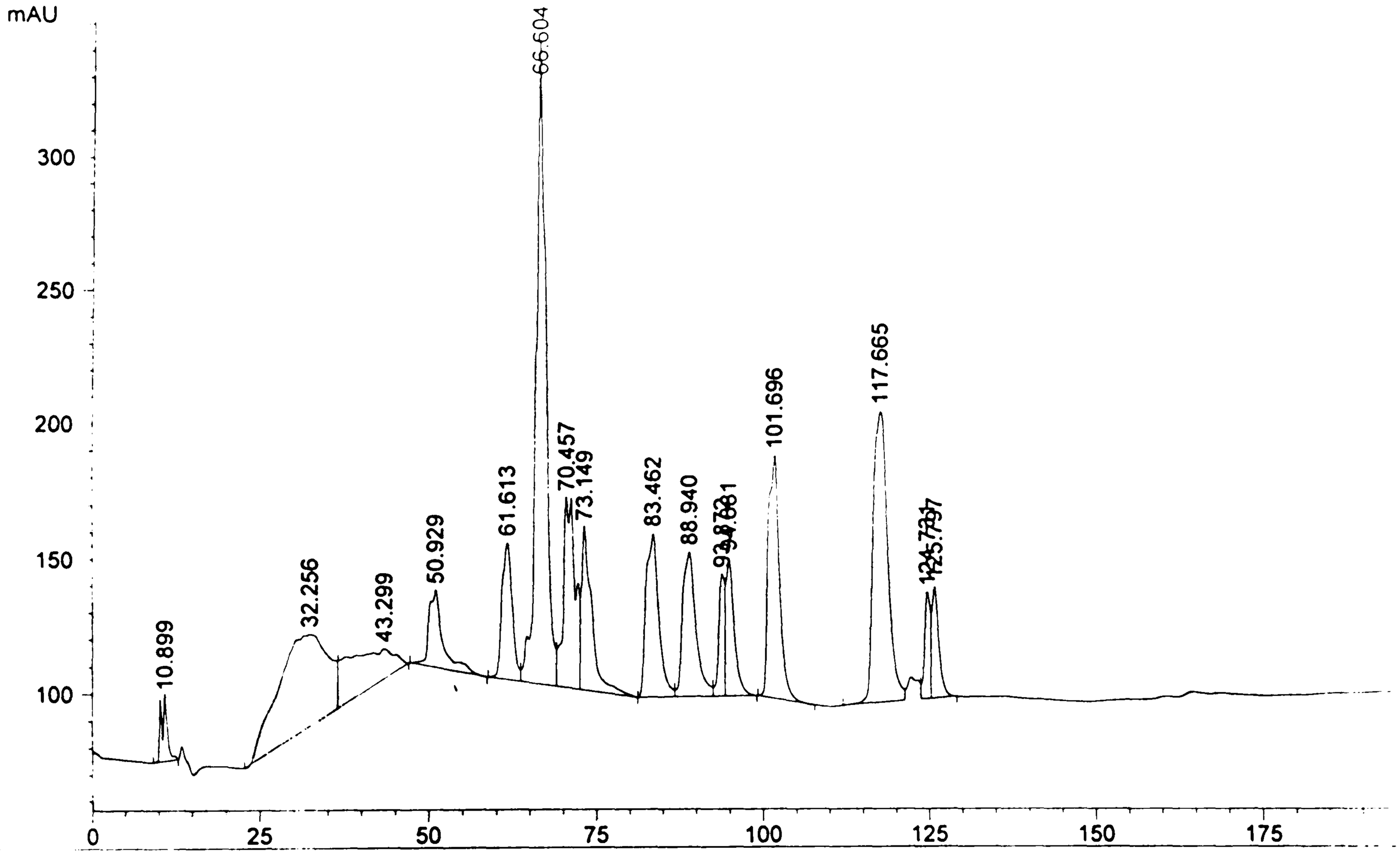
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150
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61.613
66.604
70.457
73.149
83.462
88.940
101.696
117.665

5.7.1 - HPLC separation of [^{3}H] Phenacetyl-Leu-Leu-boroLeu (radiolabelled inhibitor) modified liver 20S proteasomes

Liver 20S proteasome (130 μg; 0.8 μM) was first dialysed against 50 mM Hepes buffer/KOH, pH 7.5 and then incubated with 5 μM [^{3}H] Phenacetyl-Leu-Leu-boroLeu (radiolabelled inhibitor). This sample was incubated at 25°C for 30 minutes along with a second sample (13 μg: 0.8 μM) of dialysed liver 20S proteasome, incubated with 0.125% ethanol. This second sample was a control to measure the effect of the ethanol on the LLVY activity. Aliquots (1 μg) of the dialysed and the two preincubated 20S proteasome samples, were assayed against 40 μM Suc-LLVY-AMC as described in Chapter 2. Compared to the dialysed 20S proteasome, the LLVY activity of the ethanol and radiolabelled inhibitor treated samples were 98% and 59% respectively.

The radiolabelled 20S proteasomes were separated from the unbound radiolabelled inhibitor using a Pharmacia PD-10 column. This column run was performed as described in the legend to Figure 5.10. The 20S proteasomes were shown to elute in fractions 2 and 3 that were pooled. This 1 ml sample was prepared and subjected to HPLC separation as described in the legend to Figure 5.10. Figure 5.10 Panel A (page 185) shows the Absorbance (220 nm) against elution time (minutes) plot. The pattern of peaks were similar to previous traces obtained from the elution of 100 μg 20S proteasome. Figure 5.10 Panel B (page 186) illustrates the elution of the radioactivity during this experiment and a subsequent control run. In the control run, 0.2 μl of 1 mM radiolabelled inhibitor was prepared and separated on the C4 column, according to the standard protocol. It was anticipated that the radioactivity associated with the proteasome subunits would elute in distinct peaks. These peaks of

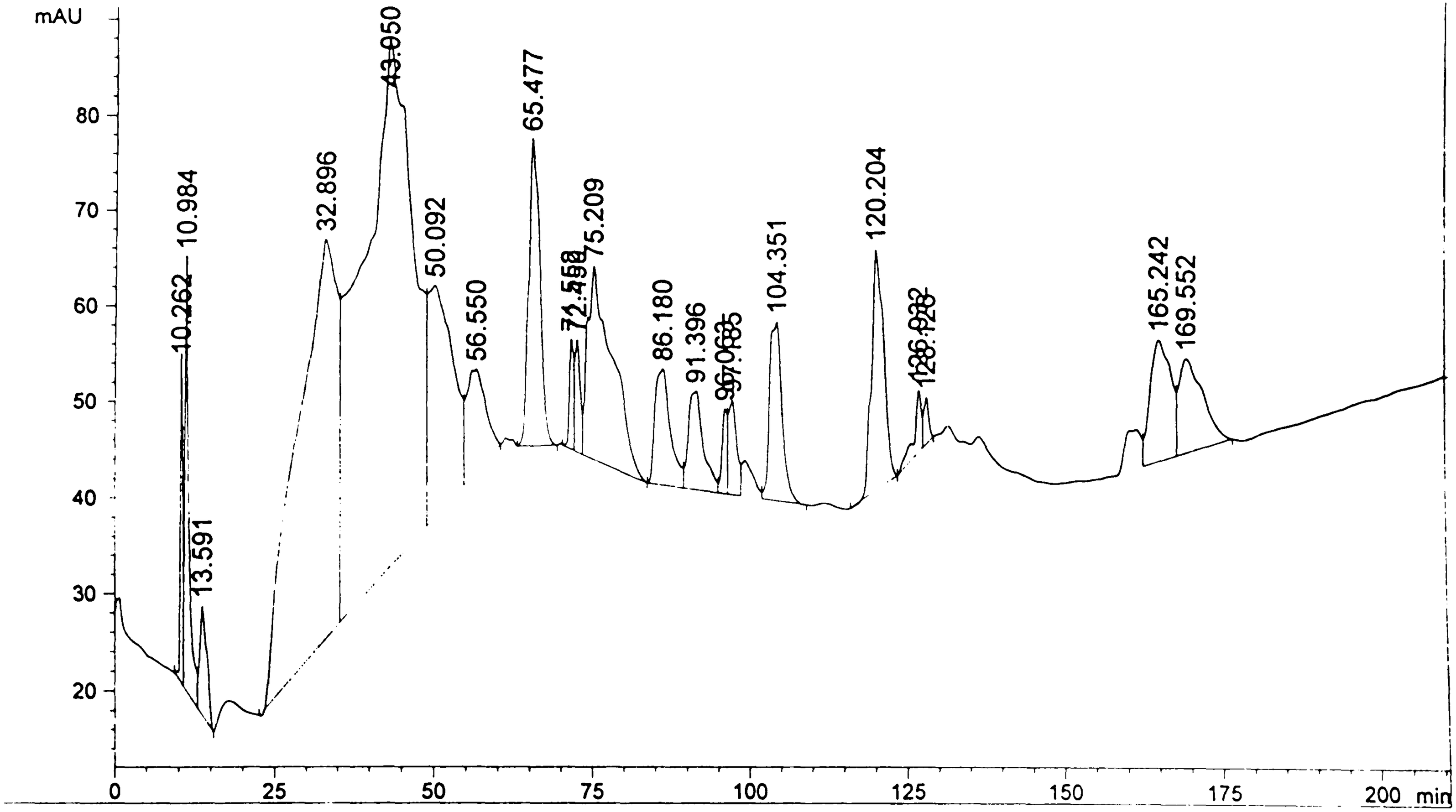
radioactivity were expected to coincide with protein peaks on the absorbance (220 nm) plot. However, the radioactivity eluted in a single broad peak. Most of the counts eluted between 49 and 99 minutes of the acetonitrile gradient with peak counts being recorded between 55 and 63 minutes. This peak did coincide with certain protein peaks on the absorbance plot. But the results suggested that the radiolabelled inhibitor had dissociated from the 20S proteasomes during preparation and separation of the subunits. The results of the control run helped to confirm this hypothesis. An aliquot of radiolabelled inhibitor (0.2 pmol) eluted from the C4 column in a single peak. The majority of the counts eluted between 50 and 70 minutes which correlates closely with the 20S proteasome separation.

To further confirm this hypothesis, HPLC fractions containing the subunit eluting at 56 minutes were pooled. This sample was run onto a Pharmacia PD-10 column as described in the legend to Figure 5.10. If radiolabelled inhibitor was still bound to the putative LMP7 subunit, the radioactivity should elute in the first 5, 0.5 ml fractions. Very low levels of radioactivity were recorded for the first eight 0.5 ml fractions. These results clearly showed that the radiolabelled inhibitor was not bound to the putative LMP7 subunit.

Figure 5.10 - Separation of [^{3}H] Phenacetyl-Leu-Leu-boroLeu (radiolabelled inhibitor) modified 20S proteasome subunits by reverse-phase HPLC

Liver 20S proteasome (150 μg; 0.8 μM) was dialysed against 50 mM Hepes buffer/KOH, pH 7.5 using a 75 kDa collodion bag. Dialysis was conducted at 4°C for 3 hours. Dialysed proteasome (130 μg) was then incubated with 5 μM [^{3}H] Phenacetyl-Leu-Leu-boroLeu (radiolabelled inhibitor) for 30 minutes at 25°C. A control sample of dialysed proteasome (13 μg; 0.8 μM) was incubated with 0.125% ethanol for 30 minutes at 25°C. Aliquots (1 μg) of radiolabelled inhibitor treated, ethanol treated or untreated 20S proteasome were assayed against 40 μM Suc-LLVY-AMC. The assays were conducted in duplicate as described in Chapter 2. Radiolabelled inhibitor and ethanol caused 41% and 2% inhibition of the LLVY activity respectively. A PD-10 gel filtration column (Pharmacia) was equilibrated with 25 ml of 50 mM Hepes buffer/KOH, pH 7.5. The radiolabelled 20S proteasome sample (250 μl) was run onto the column together with 2.25 ml of 50 mM Hepes buffer/KOH, pH 7.5. Five, 0.5 ml fractions were collected by applying 0.5 ml aliquots of 50 mM Hepes buffer/KOH, pH 7.5 to the top of the column. The protein content of the fractions was determined by the Bradford method as described in Chapter 2. The 20S proteasomes predominantly eluted in fractions 2 and 3 that were pooled. This 1 ml sample was prepared for injection into the C4 HPLC column in 4, 250 μl aliquots as described in Chapter 2. The four samples were injected into the column sequentially, washing the sample loop with 1.2 ml of 10% acetonitrile in 0.1% trifluoroacetic acid between injections. The acetonitrile gradient was run as before. Panel A (page 185) illustrates the Absorbance (220 nm) against elution time (minutes) plot. Absorbance peaks are marked with corresponding retention times in minutes. Fractions were collected every 2 minutes from 14 minutes into the gradient. Aliquots (0.3 ml) of each fraction was mixed with 8 ml of scintillation cocktail before conducting tritium counting as described in Chapter 2.

Panel B (page 186) shows the radioactivity results for this experiment (solid circle) and a control experiment (open square). In the control experiment 0.2 μl of 1 mM radiolabelled inhibitor was prepared and separated on the C4 column according to the same protocol as the 20S proteasomes.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10.984
32.896
43.050
50.092
56.550
65.477
72.458
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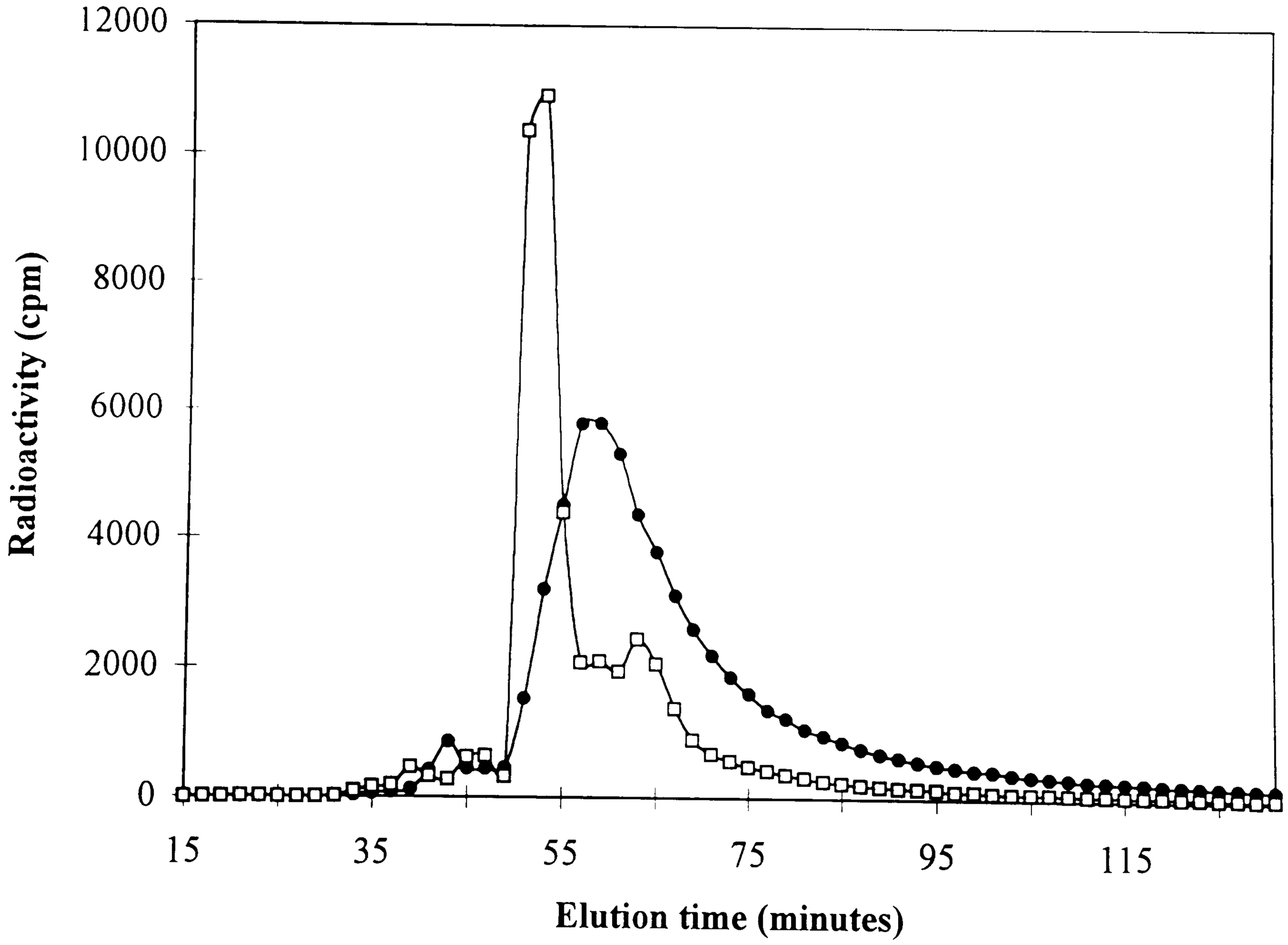
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10000
8000
6000
4000
2000
0
Radioactivity (cpm)
15
35
55
75
95
115
Elution time (minutes)

5.8 - SDS-PAGE separation of [^{3}H] Phenacetyl-Leu-Leu-boroLeu (radiolabelled inhibitor) labelled liver 20S proteasomes

3,4-dichloroisocoumarin (DCI) causes irreversible inhibition of the liver, pituitary and spleen 20S proteasome chymotrypsin-like, trypsin-like, PGPH and SNAAP activities (Djaballah et al., 1992; Orlowski et al., 1993; Eleuteri et al., 1997). The pituitary 20S proteasome BrAAP activity was strongly activated by 10 μM DCI whereas the spleen 20S proteasome BrAAP activity was not significantly effected by 20 μM DCI. Results obtained using rat liver 20S proteasomes suggested that DCI caused stimulation of trypsin-like and PGPH activities under certain conditions (Djaballah et al., 1992). SDS-PAGE was used to separate 20S proteasome subunits labelled with [^{14}C] DCI (Orlowski et al., 1997). The protein bands were transferred onto Immobilon P membranes which were washed and dried before conducting autoradiography. Six protein bands were labelled with [^{14}C] DCI. In control experiments unlabelled 20S proteasome subunits were separated by SDS-PAGE and blotted onto the Immobilon P membranes. The protein bands were sequenced to determine the migration patterns of the different β-subunits. The four intensely labelled bands 1, 2, 3 and 5 contained β-subunits X, Y and C5, RN3, and Z respectively. The β-subunits in the labelled bands 4 and 6 were not identified. The slower migrating α-subunits were not labelled.

In order to investigate whether the radiolabelled inhibitor remains bound to proteasome subunits during SDS-PAGE separation, liver 20S proteasome (150 μg) was incubated with either 2.5, 5 or 10 μM radiolabelled inhibitor for 30 minutes at 25°C. These samples were then run on a 15% SDS-PAGE gel and blotted onto nitrocellulose according to the protocol given in Chapter 2. Fluorography of the blot was performed but no clearly definable bands were

observed even after an exposure time of six months.

5.9 - Effect of Cbz-Leu-Leu-Leu-vinyl sulphone (Z-LLL-VS) on the peptidase activities of liver 20S proteasomes

Z-LLL-VS is a irreversible inhibitor of 20S and 26S proteasomes (Bogyo et al., 1997). It was proposed that preincubation of 20S proteasome with Z-LLL-VS, may block modification of the 20S proteasome catalytic sites by the radiolabelled inhibitor. In addition Z-LLL-VS could be used at a concentration that effectively inhibited only the 20S proteasome chymotrypsin-like activity. Reduction in label incorporation could then be specifically linked to reduction in modification of the chymotrypsin-like catalytic sites. Peptidyl vinyl sulphones are thought to inhibit the chymotrypsin-like activity by modifying the X/MB1 and LMP7 β-catalytic subunits (Table 1.3) (Bogyo et al., 1998). Table 5.5 describes the effect of 0-10 μM Z-LLL-VS on the LLVY activity of liver 20S proteasomes. Preincubation of 1 μg 20S proteasome with Z-LLL-VS was conducted for either 15 or 60 minutes at 37°C. The extent of inhibition was found to depend on both the concentration of inhibitor and the length of preincubation. The results in Table 5.5 are in close agreement with the results presented by Bogyo et al. (1997) using a purified mixture of 20S and 26S proteasomes. In a subsequent experiment, liver 20S proteasomes (1 μg) were preincubated with 10 μM Z-LLL-VS at 37°C for 1 hour, before assaying against four peptide substrates. The results of this experiment are shown in Table 5.6. The chymotrypsin-like activity measured with 40 μM Suc-LLVY-AMC or AAF-AMC was substantially inhibited, with the LLVY activity being inhibited to a greater extent than the AAF activity. This outcome was also observed with other types of proteasome inhibitor e.g. compound 1, diisopropyl fluorophosphate, Tyr-Gly-Arg-CH_2Cl and Z-Phe-Gly-Tyr-CHN_2

(Djaballah et al., 1992; Reidlinger et al., 1997). This difference may arise from the fact that the AAF-AMC substrate is partially hydrolysed at a second site. Z-LLL-VS (10 μM) was found to weakly inhibit the LSTR and LLE1 activities. This is in agreement with the results of (Bogyo et al., 1997). Hence at this concentration the vinyl sulphone inhibitor is primarily effective against the chymotrypsin-like activity.

Table 5.5 - Concentration and length of preincubation govern the inhibition of the liver 20S proteasome LLVY activity by Cbz-Leu-Leu-Leu-vinyl sulphone (Z-LLL-VS)

[Z-LLL-VS] (μM)	20S proteasome LLVY activity (% of control)	
	Preincubation for 15 min at 37°C	Preincubation for 60 min at 37°C
0.5	84	70
1	84	67
5	65	34
10	55	18

Assays were performed as described in Chapter 2 using 1 μg of liver 20S proteasome. The Suc-LLVY-AMC concentration was 40 μM. Values are given as the average of one experiment performed in duplicate. These values are expressed as percent of control activity in samples containing no inhibitor.

Table 5.6 - Cbz-Leu-Leu-Leu-vinyl sulphone (Z-LLL-VS) (10 μM) primarily inhibits the chymotrypsin-like activity of liver 20S proteasomes

Substrate	Activity (% of control)
Suc-LLVY-AMC	9
AAF-AMC	31
Boc-LSTR-AMC	75
Cbz-LLE-NAP	88

Assays were performed as described in Chapter 2 using 1 μg of liver 20S proteasome and a preincubation of 1 hour at 37°C. The substrate concentration was 40 μM for Suc-LLVY-AMC, AAF-AMC and Boc-LSTR-AMC. Cbz-LLE-NAP was used at 100 μM. Values are given as the average of one experiment performed in duplicate. These values are expressed as percent of control activity in samples containing no inhibitor.

5.9.1 - Inhibition of the 20S proteasome chymotrypsin-like activity by [^{3}H] Phenacetyl-Leu-Leu-boroLeu (radiolabelled inhibitor) is not easily reversed by gel filtration

Table 5.7 (page 195) illustrates the effect of the radiolabelled inhibitor on liver 20S proteasome activities before and after gel filtration. Liver 20S proteasome (80 μg at 0.05 μg/μl) was incubated with 1 μM radiolabelled compound for 15 minutes at 25°C. A control 20S proteasome solution (11 μg at 0.05 μg/μl) was also incubated for 15 minutes at 25°C. Aliquots (1 μg) of radiolabelled and control 20S proteasome solutions were then assayed with either 40 μM Suc-LLVY-AMC, AAF-AMC, Boc-LSTR-AMC or 100 μM Cbz-LLE-NAP. Both the LLVY and AAF activities were significantly inhibited by the radiolabelled inhibitor. In contrast the LSTR and LLE1 activities were stimulated by 1 μM radiolabelled inhibitor. Stimulation may have resulted from modification of chymotrypsin-like catalytic sites, causing conformational changes that stimulated activity at other catalytic sites. Also the radiolabelled inhibitor may bind to allosteric sites close to the trypsin-like and PGPH catalytic sites. These interactions may stimulate the corresponding activities. The remaining radiolabelled proteasome sample was separated from unbound radiolabelled inhibitor using two PD-10 columns. The Bradford method was used to determine the protein concentration of the fractions from the second column. Aliquots (1 μg) of 20S proteasome were then assayed against the four peptidase substrates. The inhibition of the chymotrypsin-like activity was essentially maintained during gel filtration. Cbz-Leu-Leu-boroLeu (pinacol ester) was shown to bind tightly to the 20S proteasome LLVY cleaving sites, during dialysis and immunoprecipitation experiments. Therefore it was not surprising that the closely related radiolabelled inhibitor also exhibited tight binding to the LLVY cleaving sites. The stimulation of the LSTR and LLE1 activities was mainly lost during gel filtration. Weak allosteric

interactions of radiolabelled inhibitor with proteasome β-subunits could cause the observed stimulation that was lost during gel filtration.

5.9.2 - Z-LLL-VS blocks the labelling of chymotrypsin-like catalytic sites by the radiolabelled inhibitor

Table 5.8 (page 196) shows the results of two experiments in which 10 μM Z-LLL-VS was used to block the incorporation of 1 μM radiolabelled inhibitor into liver 20S proteasomes. The vinyl sulphone proved to be an effective blocking agent, reducing incorporation of tritiated compound by 70% over two experiments. Z-LLL-VS (10 μM) inhibited the LLVY activity of 1 μg liver 20S proteasome by 87±5% (see Tables 5.5 and 5.6). Therefore 10 μM Z-LLL-VS was expected to reduce the incorporation of the radiolabelled inhibitor by approx. 85%. In the competition experiments the concentration of 20S proteasome was 10-fold higher than for the assays shown in Tables 5.5 and 5.6. At this higher concentration, the extent of modification of the LLVY cleaving catalytic sites was probably lower. This would allow the modification of the remaining sites by the more reactive radiolabelled inhibitor. It was observed that the radioactivity measured per microgram of 20S proteasome was lower than expected for the control experiments (data not shown). Tritium exchange was thought to be occuring during the gel filtration steps. Results from other experiments suggested that the level of tritium exchange was inversely proportional to the amount of radiolabelled 20S proteasome, loaded onto the PD-10 columns. The radioactivity readings for the vinyl sulphone untreated and treated samples were similar in magnitude, for both competition experiments. Because the samples contained identical amounts of 20S proteasome and were fractionated using the same protocol, it would be reasonable to expect the same level of tritium exchange

to apply to both samples. This hypothesis could be tested by repeating the competition experiment using e.g. 15 or 50 μg portions of 20S proteasome in the initial incubation tubes.

Table 5.7 - Inhibition of the liver 20S proteasome chymotrypsin-like activity by [^{3}H] Phenacetyl-Leu-Leu-boroLeu (radiolabelled inhibitor) is not readily reversed during gel filtration

Substrate	Activity (% of control) after treatment with the radiolabelled inhibitor	Activity (% of control) after removal of excess radiolabelled inhibitor
Suc-LLVY-AMC	30	43
AAF-AMC	66	61
Boc-LSTR-AMC	128	96
Cbz-LLE-NAP	253	120

Liver 20S proteasome (80 µg at 0.05 µg/µl in 50 mM Hepes buffer/KOH, pH 7.5) was incubated with 1 µM radiolabelled inhibitor for 15 minutes at 25°C. A control 20S proteasome solution (11 µg at 0.05 µg/µl in 50 mM Hepes buffer/KOH, pH 7.5) was also incubated for 15 minutes at 25°C. Aliquots (1 µg) of the radiolabelled or control proteasome solutions were assayed against either 40 µM Suc-LLVY-AMC, AAF-AMC, Boc-LSTR-AMC or 100 µM Cbz-LLE-NAP. The assays were conducted as described in Chapter 2 using a 30 minute incubation at 37°C. Two PD-10 gel filtration columns (Pharmacia) were equilibrated with 25 ml of 50 mM Hepes buffer/KOH, pH 7.5. The remaining radiolabelled proteasome sample was diluted to 2.5 ml with 50 mM Hepes buffer/KOH, pH 7.5. This sample was run onto the first column. Five, 0.5 ml fractions were collected by sequentially adding 0.5 ml aliquots of 50 mM Hepes buffer/KOH, pH 7.5 to the column. Fractions 2 and 3 were pooled and diluted to 2.5 ml with 50 mM Hepes buffer/KOH, pH 7.5. This sample was applied to the second column. Five, 0.5 ml fractions were collected as above. The Bradford method was used to determine the protein concentration of the five fractions. Aliquots (1 µg) of 20S proteasome were then assayed against the four peptidase substrates as above.

Table 5.8 - Cbz-Leu-Leu-Leu-vinyl sulphone (Z-LLL-VS) (10 μM) blocks the incorporation of 1 μM [^{3}H] Phenacetyl-Leu-Leu-boroLeu (radiolabelled inhibitor) into the chymotrypsin-like catalytic sites of liver 20S proteasomes

Experiment no.	Radiolabelled inhibitor incorporated in the absence of Z-LLL-VS (cpm)	Radiolabelled inhibitor incorporated following preincubation with Z-LLL-VS (cpm)	Reduction in radiolabelled inhibitor incorporation in the presence of Z-LLL-VS (%)
1	991	362	64
2	1090	278	75

Two Eppendorf tubes were set up containing liver 20S proteasome (27.5 μg at 0.05 μg/μl in 50 mM Hepes buffer/KOH, pH 7.5). Z-LLL-VS (10 μM) was added to tube 2 and both tubes were incubated at 37°C for 60 minutes. Radiolabelled inhibitor (1 μM) was added to both tubes that were then incubated at 25°C for 15 minutes. Four PD-10 columns were equilibrated with 50 mM Hepes buffer/KOH, pH 7.5. The contents of tube 1 (550 μl) were run into the first PD-10 column followed by 1950 μl of 50 mM Hepes buffer/KOH, pH 7.5. Five, 0.5 ml fractions were collected by applying 0.5 ml aliquots of 50 mM Hepes buffer/KOH, pH 7.5 to the top of the column. The first four fractions were pooled. This sample was run onto the second PD-10 column followed by 0.5 ml of 50 mM Hepes buffer/KOH, pH 7.5. Five, 0.5 ml fractions were collected as before. The contents of tube 2 were also fractionated using the same method and PD-10 columns 3 and 4. The Bradford method was used to determine the protein content of the fractions from PD-10 columns 2 and 4. Two, 3 μg aliquots of 20S proteasome from either PD-10 columns 2 or 4 were individually mixed with 8 ml of scintillation cocktail and subjected to radioactivity counting as described in Chapter 2. The average results from two separate experiments performed in duplicate are shown in the table.

5.10 - Summary

A number of peptidyl boronic acids were shown to traverse the plasma membrane of mammalian cells and inhibit proteasome complexes. The peptidyl boronic acids were found to bind tightly to the chymotrypsin-like active sites, with inhibition surviving either an immunoprecipitation procedure or gel filtration of a cell extract. IC50 values for the LLVY activity of 20S or 26S proteasomes immunoprecipitated from cells treated with Bz-Phe-boroLeu, were similar to Ki values for Bz-Phe-boroLeu inhibition of the same activity in purified 20S or 26S proteasomes. Ki values for peptidyl boronic acid inhibition of the liver 20S proteasome LLVY activity were also shown to be similar to IC50 values for inhibition of β-amyloid production and NF-κB activation in cultured cells. This relationship was observed with a variety of peptidyl boronic acids, displaying a large range of Ki values. [^{3}H] Phenacetyl-Leu-Leu-boroLeu labelled 20S proteasome subunits could not be separated by HPLC or SDS-PAGE, without the radiolabelled inhibitor dissociating from the subunits. Competition experiments showed that Cbz-Leu-Leu-Leu-vinyl sulphone, could block the [^{3}H] Phenacetyl-Leu-Leu-boroLeu labelling of 20S proteasome chymotrypsin-like sites. Previous studies have suggested that peptidyl vinyl sulphones inhibit the chymotrypsin-like activity of proteasomes, by modifying the amino-terminal threonines of subunits LMP7 and X/MB1.

Chapter 6 - Discussion

Chapter 6 - Discussion

6.1 - Proteasome complexes possess low specific activities compared to many other enzymes

Since the discovery of proteasome complexes, an ever increasing number of research groups have been characterising their structure, catalytic properties and the degradation of substrate proteins. Proteasome complexes are vitally important components of cellular metabolism, facilitating the degradation of many protein substrates including short-lived regulators of the cell cycle and immune responses. 20S proteasomes are unusual in possessing low specific activities compared to many other enzymes. For example the following serine endopeptidases all have specific activities that are several orders of magnitude higher than the LLVY activity of liver or spleen 20S proteasomes (10 or 12 nmol/min/mg protein):

a) α-chymotrypsin from bovine pancreas – 65 units/mg; 1 unit hydrolyses 1 μmol Suc-AAPF-4-NAP per minute at pH 7.8 and 25°C.

b) Elastase from human leukocytes – approx. 6 units/mg; 1 unit hydrolyses 1 μmol 4-nitrophenol per minute from Boc-Ala-4-nitrophenyl ester at pH 6.5 and 37°C.

c) Endoproteinase Arg-C from murine submaxillary glands – 100 units/mg; 1 unit hydrolyses 1 μmol N-tosyl-Arg-methylester per minute at pH 8.0 and 37°C.

(Specific activities obtained from the Fluka Chemika BioChemika Analytika catalogue 1997/1998 produced by Fluka Chemicals, Gillingham, England. The classification of the enzymes was obtained from Proteolytic enzymes: a practical approach, edited by R.J Beynon

& J.S. Bond (1989). IRL PRESS, Oxford).

Cysteine endopeptidases often have much greater specific activities compared to 20S proteasomes. For example:

a) Papain from Carica papaya – 20 units/mg protein; 1 unit hydrolyses 1 μmol Bz-Arg-ethyl ester per minute at pH 6.2 and 25°C.

b) Clostripain from Clostridium histolyticum – 100 units/mg; 1 unit hydrolyses 1 μmol Bz-Arg-ethyl ester per minute at pH 7.1 and 2°C.

c) Cathepsin B from bovine spleen – 10 units/mg; 1 unit hydrolyses 1 μmol Cbz-Lys-4-nitrophenyl ester per minute at pH 5.0 and 25°C.

One of the main reasons for the low specific activities of 20S proteasomes may be the unusual N-terminal catalytic threonine residues. These threonines contain a secondary hydroxyl group that is probably not as strong a nucleophile as the primary hydroxyl group of catalytic serine residues or the sulfhydryl group of catalytic cysteine residues. The N-terminal location of the catalytic threonines may have arisen to increase the nucleophilic nature of the catalytic hydroxyl group. This hydroxyl group will be close to the N-terminal α-amino group that is positively charged under physiological conditions. This positive charge will tend to repel the hydrogen nucleus of the hydroxyl and attract the electrons from the O-H bond towards the oxygen atom. This will increase the nucleophilic nature of the oxygen atom. Large complexes e.g. 19S regulatory and PA28 complexes combine with the 20S proteasome in cells. These complexes cause conformational changes that probably ease the entry of substrate molecules. The conformational changes may also facilitate the movement of residues surrounding the

catalytic threonines to increase the nucleophilic nature of the hydroxyl groups.

6.2 - Inhibitors of proteasome complexes

The peptidase activities of proteasome complexes have been studied using a wide variety of protease inhibitors and other compounds. These include peptidyl aldehydes (Rock et al., 1994. Vinitsky et al., 1992), the serine protease inhibitors 3,4-dichloroisocoumarin and diisopropyl fluorophosphate (Djaballah et al., 1992; Orlowski et al., 1997), the streptomyces metabolite lactacystin (Fenteany et al., 1995) and peptidyl vinyl sulphones (Bogyo et al., 1998). In this study, a number of di- and tripeptidyl boronic acids have been shown to be tight-binding reversible inhibitors of the 20S proteasome chymotrypsin-like activity. This inhibition is apparently exerted via a slow-binding mechanism. Slow binding inhibition has been demonstrated for the peptidyl boronic acid inhibition of certain serine proteases (Kettner et al., 1988). It has also been shown to occur in some studies investigating the inhibition of the 20S proteasome chymotrypsin-like activity by peptidyl aldehydes (Vinitsky et al., 1992). Several peptidyl boronic acids exhibited similar Ki values for inhibition of the chymotrypsin-like activity of liver and spleen 20S proteasomes. Liver 20S proteasomes contain approximately equal amounts of the constitutive and IFN-γ inducible catalytic subunits, whereas spleen 20S proteasomes are predominantly constructed with IFN-γ inducible catalytic subunits (Paul Brooks, personal communication; Eleuteri et al., 1997). Therefore these peptidyl boronic acids should cause effective inhibition of the chymotrypsin-like activity of 20S proteasomes, isolated from any cell or tissue type.

6.3 - The peptidyl boronic acids used in this study preferentially inhibited the chymotrypsin-like activity of 20S proteasomes

The peptidyl boronic acids used in this study were designed to inhibit the chymotrypsin-like activity of proteasomes and were significantly less effective inhibitors of other 20S proteasome activities e.g. trypsin-like and PGPH activities. 3,4-dichloroisocoumarin (DCI) was shown in two studies to be more effective against the chymotrypsin-like activity than the trypsin-like or PGPH activities. Under certain assay conditions the trypsin-like and PGPH activities were stimulated by DCI (Djaballah et al., 1992; Orlowski et al., 1997). Similar effects were observed with some of the peptidyl boronic acids. Lactacystin was shown to be a more potent inhibitor of 20S proteasome cleavage after hydrophobic residues compared to basic or acidic residues (Fenteany et al., 1995; Bogyo et al., 1998). A number of peptidyl vinyl sulphones exhibited a certain degree of specificity towards the chymotrypsin-like activity of proteasomes e.g. NLVS and NP-AAF-VS.

One surprising observation from this study was the fact that peptidyl boronic acids with substantially different structures, exhibited Ki values for the liver 20S proteasome chymotrypsin-like activity that were within an order of magnitude. For example Bz-Phe-boroLeu (17 nM), Cbz-nitroArg-boroLeu (pinacol ester) (39 nM), Cbz-Lys(boc)-boroLeu (pinacol ester) (116 nM) and $Ph(CH_2)_6CO$-Leu-boroLeu (pinacol ester) (116 nM). This apparent low level of specificity may be related to the observed flexibility of the 20S proteasome. Addition of the proteasome substrate Cbz-LLE-NAP (0.4 mM) caused an increase in the sedimentation coefficient of the 20S proteasome from 17.7S to 19.7S (Djaballah et al., 1993). SDS (0.01%) also caused an increase from 17.7S to 21.2S and 50 mM KCl caused a decrease to 13.9S. 26S proteasomes are known to degrade a wide variety of protein

substrates in a processive manner. The presence of multiple catalytic sites with a broad range of specificities and in particular chymotrypsin-like catalytic sites with a low level of substrate specificity, may ensure that all portions of the protein substrates are digested.

It should be noted that most of the peptidyl boronic acids used in this study were synthesized as racemic mixtures containing a mixture of R or S P1 sidechains. The obvious exception is Bz-Phe-boroLeu that was synthesized as a chirally pure (P2 sidechain S, P1 sidechain R) product. It is unlikely that both the R and S arrangements of the P1 sidechain give rise to inhibitors of equal potency. It is probably the case that one of the two isomers is the active inhibitor and therefore the Ki values for chirally pure versions of these active isomers would be reduced by a factor of 2.

More specific and effective inhibitors of the 20S proteasome LLVY activity may be produced by linking two peptidyl boronic acid functional groups together via a polar flexible linker. This bifunctional inhibitor would be capable of reacting with two catalytic sites at once. This approach has been attempted successfully using tripeptidyl aldehyde groups linked to both ends of a flexible nondegradable polyoxyethylene polymer of 19-25 monomers (Loidl et al., 1999a). An IC_{50} value of 17 nM was obtained against the chymotrypsin-like activity of yeast 20S proteasomes (Suc-LLVY-AMC substrate) using al-Nle-Leu-Leu-CO-$(PEG)_{19\text{-}25}$-CO-Leu-Leu-Nle-al. The IC_{50} values for the trypsin-like activity (Bz-FVR-AMC substrate) or the PGPH activity (Cbz-LLE-NAP substrate) were greater than 100 μM. In a similar manner al-Arg-Val-Arg-CO-$(PEG)_{19\text{-}25}$-CO-Arg-Val-Arg-al was the best inhibitor of the trypsin-like activity with an IC_{50} value of 71 nM. The heterobifunctional inhibitor al-Nle-Leu-Leu-CO-$(PEG)_{19\text{-}25}$-CO-Arg-Val-Arg-al was particularly interesting. The IC_{50} values for the chymotrypsin-like and trypsin-like activities were 31 and 97 nM respectively.

6.4 – Tri-leucine peptidyl boronic acids were the most effective inhibitors of the 20S proteasome trypsin-like activity

Cbz-Leu-Leu-boroLeu and the pinacol ester form of this inhibitor, were the most effective inhibitors of the liver 20S proteasome trypsin-like activity. In a related study Bogyo and coworkers (1998) showed that Leu-Leu-Leu-Leu-vinyl sulphone and Bz-Phe-Leu-Leu-Leu-vinyl sulphone were the most effective vinyl sulphone inhibitors of the muscle 20S proteasome trypsin-like activity. Peptidyl boronic acids with a structure similar to leupeptin (Acetyl-Leu-Leu-Arg-al) may prove to be particularly specific inhibitors of the 20S proteasome trypsin-like activity (Savory & Rivett, 1993). One research group have developed a specific inhibitor, maleoyl-βAla-Val-Arg-al of the yeast 20S proteasome trypsin-like activity (Loidl et al., 1999b). This inhibitor exhibited an IC_{50} value of 0.5 μM for the trypsin-like activity measured with the Bz-FVR-AMC substrate. Corresponding IC_{50} values for the chymotrypsin-like activity (Suc-LLVY-AMC substrate) and PGPH activity (Cbz-LLE-NAP substrate) were greater than 100 μM. Crystals of yeast 20S proteasomes soaked with maleoyl-βAla-Val-Arg-al were prepared and analysed. The aldehyde functional group was only covalently bound to the β2/Pup1 catalytic sites. In addition the maleoyl group was covalently linked via an S-C bond to Cys118 of the S3 subsite. This second covalent bond would make the inhibition practically irreversible. Asp-28 and Glu-53 appear to be particularly important specificity determinants in the S1 subunit of the β2 catalytic site (Loidl et al., 1999b, Groll et al., 1997). The mammalian subunits Z and MECL1 are homologous to the yeast β2 subunit. The Z and MECL1 subunits contain a Asp-53 and Glu-53 residue respectively (Hisamatsu et al., 1996). Both subunits contain an Asp-28 residue. Spleen 20S proteasomes that predominantly contain MECL1 subunits, have a three times lower specific activity to 40 μM Boc-LSTR-AMC, than

liver 20S proteasomes that contain approx. 50% Z and 50% MECL1 subunits. The presence of the smaller Asp-53 residue in subunit Z may ease substrate binding leading to an increase in catalytic rate. The S1 subsites of Z and MECL1 contain other polar residues e.g. Cys-31 and His-35 and this polar environment would help to explain the poor inhibitory effect of Cbz-Leu-t-butylboroLeu on the liver 20S proteasome trypsin-like activity (section 4.2.3). The nature of the residues in the S2 and S3 subsites are also very important. This is illustrated by the relative effectiveness of Cbz-Leu-Leu-boroLeu (pinacol ester) compared to Bz-Val(Ph)-boroLeu (pinacol ester) containing the large hydrophobic Val(Ph) P2 residue and $PhSO_2$-Leu-boroLeu (pinacol ester) containing the polar SO_2 as part of the group positioned in the S3 subsite.

6.5 - Bz-Phe-boroLeu is the best peptidyl boronic acid inhibitor of the liver 20S proteasome PGPH activity

Bz-Phe-boroLeu (compound 1) was the most effective of the limited number of peptidyl boronic acids tested against the liver 20S proteasome PGPH activity. Of note was the fact that 80 nM Bz-Phe-boroLeu caused significant (39%) inhibition of the liver 20S proteasome PGPH activity, whilest failing to inhibit the same activity of spleen 20S proteasomes. The β1/PRE3 catalytic subunits of yeast 20S proteasomes are proposed to catalyse the PGPH activity (Dick et al., 1998). X-ray crystallographic studies on yeast 20S proteasomes suggested that six residues are major constituents of the P1 subsite of β1/PRE3: Thr-20, Thr-31, Thr-35, Arg-45, Ala-49, Gln-53. The Arg-45 residue that is located at the bottom of the subsite is proposed to be a particularly important specificity determinant for these catalytic sites. The mammalian subunit homologs of β1/PRE3 are Y/δ and LMP2. The six important residues are conserved

in Y/δ but only Ala-49 and Gln-53 are conserved in LMP2. The four changes are listed below:

Residue	Y/δ	LMP2
20	Thr	Val
31	Thr	Phe
35	Thr	Ser
45	Arg	Leu

These changes will increase the hydrophobic nature of the LMP2 S1 subsite. These and other changes in the S2 and S3 subsites may explain the decreased affinity of Bz-Phe-boroLeu for the PGPH catalytic sites of spleen 20S proteasomes. The increased hydrophobicity of the S1 subsite will presumably inhibit the binding of the Glu residue in Cbz-LLE-NAP, thus helping to explain the decrease in specific activity of spleen 20S proteasomes compared to liver 20S proteasomes.

6.6 - Peptidyl boronic acids are useful tools for studying biochemical processes that involve protein degradation

A number of classes of proteasome inhibitor have been used in cell culture experiments. Peptidyl aldehydes e.g. Cbz-Leu-Leu-norVal-al and Cbz-Leu-Leu-Leu-al have been used to study the role of proteasomes in producing antigenic peptides for presentation on MHC class I molecules (Rock et al., 1994; Grant et al., 1995; Hughes et al., 1996; Vinitsky et al., 1997). They have also helped to elucidate the role of proteasome complexes in degrading IκBα and the role of the human cytomegalovirus protein US11, in stimulating the degradation of MHC class I heavy chains by the 26S proteasomes (Alkalay et al., 1995; Wiertz et al., 1996b). The

degradation of the latter substrate was proposed to be performed on the cytosolic face of the endoplasmic reticulum. However, peptidyl aldehydes are readily reversible inhibitors that need to be used at micromolar concentrations in these cell culture experiments. They also inhibit calpains and lysosomal cysteine proteases e.g. cathepsin B (Rock et al., 1994). The streptomyces metabolite lactacystin inhibits cell cycle progression and the production of antigenic peptides (Fenteany et al., 1995; Craiu et al., 1997). Experiments using [^{3}H] lactacystin showed that the only radiolabelled cell components were proteasome complexes (Fenteany et al., 1995). Although lactacystin is cell permeable and apparently very specific, it is routinuely used at micromolar concentrations making the modification of other proteases more likely. For example a cathepsin A-like activity was significantly inhibited by 1-5 μM lactacystin (Ostrowska et al., 1997). This inhibition was dependent on the preincubation of lactacystin at pH 8.0 to allow production of the β-lactone species, before conducting the assays at pH 5.5.

Peptidyl boronic acids will be a useful alternative to peptidyl aldehydes, lactacystin and peptidyl vinyl sulphones in cell culture experiments. These inhibitors cause significant inhibition of 20S and 26S proteasomes at nanomolar (e.g. 10-100 nM) concentrations. Adams and coworkers (1998) determined the Ki for inhibition of the 20S proteasome LLVY activity by 2-pyrazinylcarbonyl-Phe-boroLeu to be 0.62 nM. The corresponding Ki values for inhibition of the serine proteases human chymotrypsin and human cathepsin G were 320 and 630 nM respectively. In this study, I have shown that proteasome complexes are the only tight-binding cellular target for 100 nM [^{3}H] Phenacetyl-Leu-Leu-boroLeu. When using 1 μM [^{3}H] Phenacetyl-Leu-Leu-boroLeu, an additional small peak of radioactivity was observed in fraction 17 (Figure 5.3). This peak corresponded with a peak of Suc-LLVY-AMC hydrolysing activity, observed in assays conducted in the absence of SDS. The nature of these additional

target(s) was not investigated. One or more of these targets could be the ubiquitously expressed μ- and m-calpains. These Ca^{2+}-dependent cysteine proteases have been proposed to play roles in integrin-mediated signal transduction and cell cycle control (Arthur et al., 1998). Previous studies have shown that calpain inhibitor I (acetyl-Leu-Leu-Nle-al) and calpain inhibitor II (acetyl-Leu-Leu-Met-al) inhibit the chymotrypsin-like activity of 20S proteasomes (Vinitsky et al., 1992). Therefore inhibition of calpains by closely related peptidyl boronic acids is a likely occurrence in cells. Antibodies to μ- and m-calpains are commercially available and these could be used to determine whether calpains are a target of [^{3}H] Phenacetyl-Leu-Leu-boroLeu.

Peptidyl boronic acids are useful for studying the degradation of individual proteins. The degradation of endogenously expressed glucocorticoid receptor has been shown to be inhibited by Bz-Phe-boroLeu in Rat-1 fibroblast cells (Tavner, S. & Mason, G.G.F., unpublished observations). The nuclear localisation signal of the receptor is masked by the binding of the molecular chaperone HSP90. The binding of an appropriate steriod hormone causes the release of HSP90 and the exposure of the nuclear localisation signal. The steriod hormone-glucocorticoid receptor complex then translocates into the nucleus, to activate transcription of specific genes (Alberts et al., 1994). Bz-Phe-boroLeu caused a dose-dependent accumulation of the glucocorticoid receptor. The results in Table 5.4 show that a number of peptidyl boronic acids inhibit the production of β-amyloid in cultured cells. The Ki values for inhibition of the LLVY activity of purified 20S proteasomes compared well with the IC50 values for inhibition of β-amyloid production and NF-κB activation in cultured cells. 26S proteasomes are thought to stimulate the production of active p50-p65 NF-κB complexes, through degrading the inhibitory IκB proteins and catalysing the co-translational processing

reaction required for the production of mature p50 subunits (Alkalay et al., 1995; Lin et al., 1998b). Inhibition of proteasome complexes by peptidyl boronic acids is thought to directly or indirectly inhibit the γ-secretase activity that is required for production of β-amyloid. The production of p3 by the α- and γ-secretases is also inhibited by the peptidyl boronic acids (Christie et al., 1999). In contrast the peptidyl boronic acids have little effect on the production of sAPPα produced by the α-secretase or the soluble APP protein produced by the β-secretase.

6.7 - Determining the peptidyl boronic acid modified catalytic subunits of 20S proteasomes

A number of experiments were carried out to attempt to determine which catalytic β-subunits were modified by Cbz-Leu-Leu-boroLeu and [^{3}H] Phenacetyl-Leu-Leu-boroLeu. Unfortunately the association of these peptidyl boronic acids with the catalytic residues e.g. catalytic N-terminal threonine, did not survive the conditions required to separate the subunits by HPLC or SDS-PAGE. However, competition experiments showed that Z-LLL-VS could block binding of [^{3}H] Phenacetyl-Leu-Leu-boroLeu to the chymotrypsin-like catalytic sites. Studies with a number of peptidyl vinyl sulphones showed that the catalytic subunits X/MB1 and LMP7 predominantly hydrolyse the chymotrypsin-like substrates (Bogyo et al., 1998). PAL peptidyl boronic acids contain a reactive group e.g. phenylazide or benzophenone that is activated by U.V. light. The PAL peptidyl boronic acids were found to bind tightly to the chymotrypsin-like catalytic sites of 20S proteasomes, during dialysis experiments. U.V. irradiation could be used to facilitate the production of a separate covalent bond between the PAL peptidyl boronic acid and the residues surrounding the catalytic site. This covalent bond

may survive HPLC separation of the 20S proteasome subunits. Catalytic subunits e.g. X/MB1 or LMP7 could then be dialysed against a suitable buffer before digestion with trypsin. The tryptic fragments would then be separated using a C18 HPLC column. A shift in the elution position of a peptide, when compared to the peptide map of an unmodified subunit, would indicate PAL peptidyl boronic acid modification of this peptide. Sequencing of the appropriate unmodified and modified peptides could then be used to identify the modified residue(s). These residues would not necessarily be the catalytic threonine because the photoactivatable group is located away from the boronic acid functional group.

6.8 - Use of inhibitors to determine the roles of the various proteasome complexes in protein degradation

Highly effective inhibitors of proteasomes may facilitate a greater understanding of how the various proteasome complexes operate together in cellular protein degradation. The PA28 complex is known to stimulate the chymotrypsin-like, trypsin-like and PGPH activities of 20S proteasomes (Kuehn & Dahlmann, 1996). Binding of the PA28 complex to the 20S proteasome causes an increase in the diversity of peptides produced, during the digestion of synthetic 25 amino acid peptides (Groettrup et al., 1995). Very effective peptidyl boronic acids are useful tools because they open up the possibility of differentially inhibiting proteasome complexes. For example a number of peptidyl boronic acids may be very effective against the 20S proteasome and PA28-20S proteasome activities but not against the 26S proteasome. This will help investigators to determine whether the peptides produced by the 26S proteasome, are often trimmed before being incorporated into MHC class I molecules. Specific inhibitors of hybrid proteasome activities would enable researchers to determine the role of this complex in protein degradation. A specific inhibitor of PA28-20S proteasome

activities e.g. chymotrypsin-like activity could also be used to ascertain whether any protein substrates are only degraded by the PA28-20S proteasome. This degradation would probably occur with the assistance of molecular chaperone molecules to unfold the protein before degradation.

6.9 - Use of peptidyl boronic acids as therapeutic agents

Recently reported studies by scientists at Proscript Inc. (Palombella et al., 1998; Grisham et al., 1999) showed that 2-pyrazinylcarbonyl-Phe-boroLeu (PS-341) and another dipeptidyl boronic acid named PS-273, could inhibit the degradation of IκBα and the activation of NF-κB in cultured cells. The expression of proinflammatory mediators such as cell surface adhesion molecules and cytokines could be blocked by either of these compounds. In further in vivo studies polyarthritis was induced in lewis rats by injection of a peptidoglycan/polysaccharide solution, derived from Group A streptococcal bacterial cell walls (Palombella et al., 1998). Rats received either 0 or 0.3 mg/kg 2-pyrazinylcarbonyl-Phe-boroLeu by oral administration each day from day one of the 28 day trial. The peptidyl boronic acid was shown to substantially reduce the redness, swelling and deformity of joints, even if the administration of the compound was delayed for 7 days. Histological analysis of the joints from untreated arthritic, 2-pyrazinylcarbonyl-Phe-boroLeu treated arthritic and normal healthy rats showed that the compound prevented many of the characteristic changes associated with the polyarthritis (Palombella et al., 1998).

In a recent study, 10 μM Cbz-Leu-Leu-Leu-al was shown to promote the apoptosis of chronic lymphocytic leukemic (CLL) lymphocytes (Chandra et al., 1998). Significant increases in the rate of cell apoptosis were observed with CLL lymphocytes that (i) exhibited relatively high

basal levels of apoptosis, (ii) exhibited low basal apoptosis levels that were strongly increased in response to glucocorticoid treatment and (iii) exhibited low basal and low glucocorticoid induced levels of apoptosis. Similar results were obtained with lactacystin. Further experiments showed that Cbz-Leu-Leu-Leu-al caused an increase in caspase activity. It was proposed that Cbz-Leu-Leu-Leu-al drastically reduced the production of active NF-κB by inhibiting the 26S proteasome. Lack of active NF-κB was suggested to lead to a reduction in mitochondrial membrane potential, release of cytochrome c and caspase activation. Cbz-Leu-Leu-Leu-al (10 μM) was shown to cause a 2-fold lower level of apoptosis in normal lymphocytes with T-cells being most effected (Chandra et al., 1998). 20 μM Cbz-Leu-Leu-Leu-al was shown in a separate study to induce apoptosis in a prostate carcinoma cell line (Lin, et al., 1998a). 2-pyrazinylcarbonyl-Phe-boroLeu (PS-341) has recently been reported to possess antitumour activity (Adams et al., 1999). This peptidyl boronic acid was shown to inhibit the growth of 60 different tumour cell lines at low e.g. 7 nM concentrations. Further studies suggested that PS-341 caused the accumulation of the cyclin-dependent kinase inhibitor protein p21, in the PC-3 prostate tumour cell line. This lead to the inhibition of cyclin-dependent kinase 4 and the arrest of the cell cycle at the G_2-M transition. PS-341 was shown to stimulate caspase activity and apoptosis in growth-arrested tumour cells. Direct injection of PS-341 into the blood stream of tumour-bearing mice at a level of 1.0 mg/kg body weight, once weekly for 4 weeks caused an approximately 60% decrease in tumour volume. This level of PS-341 treatment was apparently well-tolerated by non-tumour cells with no adverse effects being noted. [^{14}C] PS-341 was shown to concentrate in the adrenals, kidney cortex, liver, prostate and spleen. Low levels of radioactivity were observed in e.g. skin and muscle tissue with very little penetration of the radiolabelled inhibitor into brain, spinal cord, eye or testes tissues.

Peptidyl boronic acids are important new drugs in the treatment of various forms of cancer and the effects of arthritis. Further studies on elucidating the exact mechanisms by which tumour cells are stimulated to go into apoptosis or the symptoms of arthritis are elevated may guide scientists towards new strategies for treating these and other diseases.

Publications arising from this work and references

Publications arising from this work

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